An investigation into the antimicrobial activity of thiomersal and its photodegradation products.

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AN INVESTIGATION INTO THE ANTIMICROBIAL ACTIVITY OF THIOMERSAL AND ITS PHOTODEGRADATION PRODUCTS

THESIS

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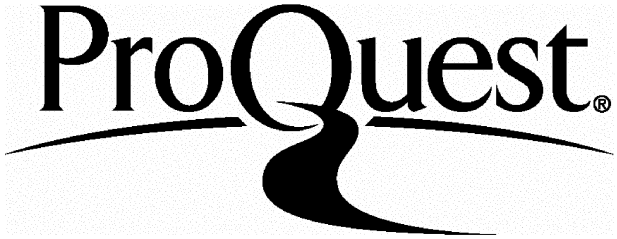
This research was carried out in the School of Pharmacy and Pharmacology of the University of Bath, under the supervision of D.J.G. Davies, M.Sc., Ph.D., F.P.S. and B.J. Meakin, B.Pharm., F.P.S.



To Mike, Mother, Father and Yvette

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SUMMARY

The influence of experimental conditions on the antimicrobial activity of thiomersal has been studied in this work. Of these, factors such as temperature, pH, composition of test solution and age of test culture were demonstrated to have a marked effect on the activity of this organic mercurial, and served to emphasise the need for standardisation of methods for determining the efficiency of preservatives.

Photochemically degraded solutions of thiomersal were demonstrated to have an increased antimicrobial activity. This is especially evident with Ps. aeruginosa, where with increased photodegradation, a concomitant increase in activity was obtained, when solutions were prepared in isotonic Sörensen's phosphate buffer, pH 7.0. Subsequently, EDTA was included in formulations to determine if the enhanced activity observed was related to chelateable ions. This was indeed shown to be the case, as in the presence of EDTA, a reduction in antimicrobial activity was observed, although in all cases, degraded solutions were still more effective than their undegraded counterpart.

An HPLC system was developed to study the composition of degraded solutions. This gave good separation of thiomersal from its degradation products and was not affected by the presence of EDTA in a formulation. The numbers of degradation products obtained made it impossible to attempt to relate a particular product or variation in peak height with observable enhanced antimicrobial activity. Chromatograms of test solutions revealed that no breakdown of undegraded thiomersal could be detected after microbial challenge experiments. When degraded solutions from microbial challenge experiments were chromatographed,

however, it became evident that the micro-organisms had degraded these further, possibly in the search for suitable carbon sources.

CONTENTS

LIST OF FIGURES

LIST OF TABLES

ORIGIN AND SCOPE

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Mercurial compounds have been used to kill micro-organisms for many years. Initially, the inorganic compounds were used exclusively although their tissue irritancy and extreme toxicity in some cases, were always a serious drawback. A search was, therefore, initiated for organic mercurials with good antimicrobial activity and low concomitant toxicity. Thiomersal was first used in this context in the early 1930's and has been widely used up to the present time. Despite this, little is known about its precise mechanism of action and there is still disagreement about its efficiency as an antimicrobial agent. Some of this confusion may have arisen because of the instability of thiomersal. Previous work in this School had indicated that aqueous solutions of thiomersal could undergo photodegradation and that such solutions had an improved antipseudomonal activity due to a product or products formed.

It was, therefore, decided to carry out an investigation into the antimicrobial activity of thiomersal, both undegraded and photochemically degraded, and the following line of study was envisaged:-

1) the antimicrobial activity of thiomersal under carefully controlled conditions would be studied, and the effect of varying parameters on this established,
2) aqueous solutions of thiomersal would be subjected to photodegradation and used in antimicrobial studies; a high pressure liquid chromatography system would then be used to analyse such solutions, before and after microbial challenge experiments; in so doing, it was hoped that some information on the compound(s) involved would be made available,
3) radiolabelling studies were to be undertaken as it was hoped that by selectively labelling the atoms of thiomersal, an

insight would be gained into the long 'shoulder' periods characteristic of thiomersal survivor curves.

INTRODUCTION

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The growth of micro-organisms in a pharmaceutical product may result in undesirable physical or chemical changes such as alterations in colour, taste, odour, pH, viscosity or emulsion stability (1, 2). These changes arise because certain micro-organisms are able to utilise the drug itself or the formulatory adjuvants as nutrient sources (3-7). Such microbial degradation can be an important cause of the instability of drugs, and where formulations are given by specific routes, for example, in injections or as ophthalmic products, there is the additional hazard of an infection occurring. The risk is highest with multi-dose preparations, as repeated usage and poor hygiene can easily result in the introduction of a contaminant. The need for preservation is, therefore, twofold:-

1) to ensure the maintenance of potency and stability of a preparation in the event that microbial contamination is inadvertantly introduced,
2) with certain routes of administration, to protect the consumer from infection should such contamination occur.

Preservatives should not, however, be used as an alternative to good manufacturing practice. They should instead, be considered as built-in safeguards against chance contamination, inadvertently introduced subsequent to the manufacturing process.

Antimicrobial agents may be thought of as being 'bactericidal' or 'bacteriostatic', according to whether they are used to kill bacteria or merely inhibit their growth (8). Most antimicrobial agents show both bactericidal and bacteriostatic activities; features which can be concentration dependent. It has been suggested (9-14) that an ideal preservative should possess as many as possible of the following attributes, and thus be:-

1) active against a wide range of micro-organisms,
2) soluble in water or readily miscible with the formulation,
3) non-toxic, non-irritating, non-sensitising and non-allergenic, both externally and internally, at the required concentration,
4) compatible with a wide range of drugs and formulatory adjuvants,
5) active and stable over a wide range of pH,
6) stable at temperatures used during manufacture, as well as during the shelf-life of the preparation,
7) free from any objectionable colour or odour, or otherwise affect the appearance of the preparation,
8) not subject to interaction with the container or sorption by rubber or plastic,
9) relatively unaffected by the presence of extraneous organic matter,
10) readily available and low in cost.

As well as the above, the following criteria also have relevance:-

1) rapidity in action. If a multi-dose ophthalmic preparation becomes contaminated during use, it is important that the preservative is able to re-sterilise the product quickly. Ideally, this re-sterilisation should be achieved within one hour (14, 15). This is because, despite the fact that a solution may contain a preservative, if it is used again before sufficient time has elapsed for all the organisms to be destroyed, living organisms can find their way through any abrasion in the corneal stroma, and so initiate an infection.
2) concentration exponent. This term is frequently associated with disinfectants, but also has significance when dealing

with preservatives. The relationship between concentration and activity can be expressed as shown in the following equation:-

$$C^n t = \text{constant} \qquad (1)$$

where C is the concentration of disinfectant, t is the time taken to kill the test culture, and n is the concentration exponent (16). n can be estimated either graphically, (by plotting the log of the time required to kill a standard inoculum against the log of the concentration and determining the slope of the straight line obtained), or by substitution in the equation below:-

$$n = \frac{\log t_2 - \log t_1}{\log C_1 - \log C_2} \qquad (2)$$

where t_1 and t_2 are the kill times at concentrations C_1 and C_2 respectively.

A knowledge of n will indicate whether a disinfectant can easily be diluted out, i.e. it has a high concentration exponent, or whether dilution has a marginal effect on activity, as in the disinfectant with a low concentration exponent. Knowing the n values for preservatives is important. A preservative with a high concentration exponent should not be used in situations where a drop in concentration could quite easily occur, for example, by sorption into the plastic containers of contact lens solutions (17).

There is no single preservative known that will satisfactorily meet all the above requirements. The criteria are in fact unrealistic, as biological activity always appears to be associated with a degree of tissue irritancy or toxicity. Selection of a preservative must,

therefore, be based on knowledge of a formulation, and an appreciation of the capabilities of acceptable antimicrobial agents. By tailoring a potential preservative for a particular product in this way, a preservative might be found to be satisfactory in the context in which it is used.

Preservation of Ophthalmic Preparations

The ophthalmic preparations form some of the major pharmaceutical products which routinely incorporate preservatives. There have been several reports of corneal ulcers, resulting in the loss of sight in some cases, associated with the microbial contamination of such products (18-25). The natural protective mechanisms of the eye include blinking and the sluicing effects of the lachrymal secretions, together with the presence of lysozyme from tears. The unbroken corneal epithelium provides an efficient barrier against invasion by micro-organisms, but injuries to the cornea can occur very easily through incised wounds, foreign bodies, thermal and chemical burns (26). Abrasions can also occur during tonometry and through the use of badly fitting contact lenses. The best made contact lenses still represent 'foreign bodies' to the eye, and will interrupt the normal tear flow and washout procedures to a limited extent, as well as increase the possibility of an abrasion occurring to the cornea (27). Sometimes, a small abrasion may remain undetected, and the instillation of contaminated eye-drops can result in bacteria penetrating the underlying stroma, which is non-vascular and an excellent culture medium. Any bactericide present may be inactivated by tissue components and the rapid growth of pathogenic organisms can quickly lead to corneal ulceration and loss of sight in some cases (26).

The need for special care in the preparation of eye-drops has long been appreciated. The monograph of the 1907 British Pharmaceutical Codex (B.P.C.), specified that boiled and cooled distilled water be used. By 1911, this has been amended to stipulate that freshly sterilised and cooled distilled water be used. In 1934, the B.P.C. carried the recommendation that the apparatus, containers and solution be sterile. By 1959, eye-drops were directed to be freshly prepared, using aseptic precautions; they were also required to be dispensed in previously sterilised containers. The 1963 B.P.C. continued the trend of introducing more stringent requirements and specified three methods of sterilisation, namely, heating in an autoclave, bacterial filtration and heating with a bactericide in the final container for thirty minutes at 98-100^{o}C. Eye-drops prepared by the latter method had to be freshly made. Since then, there has been little change to these requirements.

Preservatives are only contra-indicated in ophthalmics, where solutions are intended for instillation or injection into the chamber of the eye, as during intra-ocular surgery. In these cases, solutions are sterilised by autoclaving and packaged in sterile, single-use or single-dose containers. Similar precautions are also recommended with ophthalmic solutions that are to be instilled into the conjunctival sac of injured eyes, to avoid the possibility of irritating the lining of the anterior chamber of the eye (14). With these exceptions, all ophthalmic solutions contain preservatives.

Bacteria are by far, the commonest cause of the contamination of ophthalmic products (28, 29), and of the bacteria that can cause serious damage to the eye, Pseudomonas aeruginosa is the most notorious (18-22, 24, 25, 30-33). This organism is particularly dangerous because it is

pathogenic and exceptionally resistant to antibacterial and chemotherapeutic agents (20, 32). It is widely distributed in nature, can tolerate a broad range of temperatures (32), and has simple nutritional requirements (20). Laboratory stocks of distilled water are frequent causes of contamination with this organism (26). In addition, it has been shown to grow in simple salt solutions, such as normal saline, and utilise the components of many typical ophthalmic solutions (4, 5, 19). Fisher and Allen (34) showed that Ps. aeruginosa could produce an enzyme that degraded corneal collagen in rabbits. Furthermore, it has been demonstrated that the presence of about 75-100 organisms was sufficient to cause ulcers when injected into the corneas or introduced into the incised eyes of laboratory rabbits (35, 36).

Staphylococcus aureus, Proteus vulgaris and Alkaligenes faecalis have also been isolated from infected eyes and contaminated solutions (19, 20, 23, 24, 26, 33). Staph. aureus is reputed to cause about 90% of eye infections (37); Bacillus subtilis may produce a serious abcess when it infects vitreous humour (14). Fungi and yeasts have only rarely been isolated either from contaminated preparations (29), or in infections of the eye (33, 38); the main problems here can be caused by Aspergillus fumigatus and Candida albicans.

Types of Preservatives used in Ophthalmic Preparations

Quaternary Ammonium Compounds. Benzalkonium chloride is the most commonly used antimicrobial agent in ophthalmic solutions. It is normally used in eye-drops at a concentration of 0.01% w/v, and in hard lens solutions at concentrations of 0.001-0.01% w/v (27). Many commercial formulations often contain this preservative in combination with other agents, especially ethylenediaminetetraacetic acid (EDTA).

It is widely compatible with other antimicrobial agents, but incompatible with anionic drugs, salicylates, nitrates and non-ionic surfactants in high concentrations.

It has been demonstrated that a 0.02% solution killed an inoculum of 10^8 cells ml^{-1} of 13 strains of Ps. aeruginosa in 45 minutes, whereas a 0.01% solution required up to 9 hours (15). The inactivating medium used here contained Tween 80 and lecithin. Other workers (39) have found that benzalkonium chloride was much more effective against Ps. aeruginosa and they determined that a 0.01% w/v solution took about a minute to reduce the original count of 10^6 organisms ml^{-1} by 5 log cycles. These differing results, apart from being obtained under different experimental conditions, may reflect the variable response of strains of Ps. aeruginosa to benzalkonium chloride.

Other quaternary compounds used in ophthalmic solutions include benzethonium chloride at 0.025% and cetylpridinium chloride at 0.02%.

Substituted Alcohols. Chlorbutol is stated to be effective against both Gram-positive and Gram-negative organisms, including Ps. aeruginosa (14). Its effective concentration is 0.5%, but as this is fairly close to its saturation point, in cold weather, crystals may be deposited. It is volatile, unstable in solutions of pH 6 and above, but relatively stable below pH 6 (26). It is generally compatible with other ophthalmic components, but is sorbed by polyethylene and polypropylene containers (17).

Lawrence (9) found that a 0.5% solution killed heavy inocula of 26 strains of Ps. aeruginosa and 4 species of Proteus in simple buffer, in contact times of under half and up to 48 hours at 24-26°C. His recovery media did not contain an inactivator for chlorbutol, as he maintained that there was a minimal difference between its bactericidal and

bacteriostatic activities. The evidence for this statement is not provided, so that his data must be treated with reservation.

Other substituted alcohols include phenylethyl alcohol (PEA), which is normally used at a concentration of 0.5%. Kohn, Gershenfeld and Barr (15) tested a 0.5% PEA solution against 13 strains of Ps. aeruginosa (10^8 cells ml^{-1}), using Tweens as inactivating agents in their recovery media. Killing was not achieved despite a 24-hour contact time, and they concluded that it was a poor preservative against this organism at the concentration used. Hugo and Foster (40) found that at 18°C, a 0.6% w/v solution of PEA killed an inoculum of 100 organisms ml^{-1} of a strain of Ps. aeruginosa in 45 minutes; a concentration of 0.9% w/v was required to kill the same inoculum size of the identical strain in 30 minutes. Control experiments showed that inactivation had been achieved in the media they used. They supported the view of Klein, Millwood and Walther (41), that PEA should be used at concentrations greater than 0.5% w/v. Riegelman, Vaughan and Okumoto (35), in preliminary studies on PEA, had found that concentrations greater than 0.75% were too irritating to the eye, however, so that increasing its concentration would not be without problems.

The best current application of PEA appears to be with the established antibacterial agents, to enhance their activity against Ps. aeruginosa. In this context, it has been successfully shown to promote the activity of benzalkonium chloride, chlorhexidine, chlorbutol, chlorocresol, phenylmercuric nitrate, thiomersal and a hydroxybenzoate mixture (42-47). Its effect is apparently exerted by altering the permeability properties of Gram-negative cells (42).

Parahydroxybenzoic Acid Esters. The esters of parahydroxybenzoic acid are more usually known as parabens. Mixtures of methylparabens (0.0229%)

and propylparabens (0.0114%) were used in 'Solution for Eye-Drops', until this preparation was replaced by chlorocresol in the B.P.C. 1963; subsequently, the solution was re-introduced. They have a slow anti-microbial action and have been used as a carbon source at the concentration recommended in 'Solution for Eye-Drops' by Ps. aeruginosa (48) and other micro-organisms (49). In water and at the recommended concentrations, they have also been shown to support the growth of moulds (11). Hugo and Foster (40) showed that at 18°C, a strain of Ps. aeruginosa (100 organisms ml^{-1}), was hardly affected by the concentration of esters used in 'Solution for Eye-Drops'. Lawrence (9) found that with a mixture of methylparabens (0.16%) and propylparabens (0.02%), kill times of 1-6 hours were obtained for heavy inocula of 26 strains of Ps. aeruginosa and 4 species of Proteus. No inactivating substance was used in his recovery media for parabens, however, on the reasoning that there was no appreciable difference between their bactericidal and bacteriostatic activities. Once again, no evidence for this statement was provided, so that the reasoning and hence results, must be treated with reservation.

Chlorocresol. Chlorocresol is an established antibacterial agent. Klein et al (41) showed that at less than 0.1%, chlorocresol was able to rapidly kill an inoculum of about 10^8 cells ml^{-1} of 3 strains of Ps. aeruginosa at 37°C. The exact time taken was not determined. They also tested both 0.03% and 0.1% chlorocresol in the presence of 3 eye-drop preparations, against an 18-hour culture of Ps. aeruginosa (10^8 organisms ml^{-1}). With 0.03% chlorocresol, the atropine drops were sterilised within 4 hours, whilst the fluorescein and eserine drops required between 6 and 21-24 hours. With 0.1% chlorocresol, sterility was achieved in less than an hour with all 3 eye-drops. They concluded that the 0.1% solution was effective, but reported that this

concentration did cause some smarting. Hugo and Foster (40) found that 0.05% w/v chlorocresol could kill 10 organisms ml^{-1} of a strain of Ps. aeruginosa within 30 minutes at 18°C; 0.07% w/v was required to kill 100 organisms ml^{-1} within the same time. A 0.1% solution was found to kill 100 organisms ml^{-1} of the same strain in 10 minutes at 18°C. Control experiments previously carried out showed that their inactivating methods were effective. As chlorocresol has a high concentration exponent, small changes in concentration cause relatively large differences in the kill times obtained.

The B.P.C. withdrew chlorocresol as a preservative for eye-drops and reverted to recommending 'Solution for Eye-Drops' as a result of a report of damage to the eye, incurred not as a result of the instillation of eye-drops, but as intra-ocular injection of normal saline containing 0.1% chlorocresol. Despite the fact that the concentration of chlorocresol used was twice that recommended for eye-drops, and that the preparation should not have been used at all under those circumstances, its use was discontinued. Later in the same year, it was shown that a 0.05% solution in normal saline caused the cornea to become opaque when injected into the anterior chamber of a rabbit's eye (50). Whilst this finding might appear to support the decision of the B.P.C., it would not apply to solutions used with intact eyes.

Chlorhexidine. This has gained wide usage as a result of its rapid action and its broad spectrum of activity, both the diacetate and digluconate salts being used (51). Davies felt that chlorhexidine was the preservative of choice for use with hydrophilic soft contact lenses in terms of efficiency, stability and general tissue tolerance (27). A 0.005% solution of chlorhexidine was shown not to affect the cornea when injected into the anterior chamber of a rabbit's eye (50).

Davies, Richardson, Norton and Meakin (52) showed that chlorhexidine gluconate in concentrations of 0.001%, 0.004% and 0.008% w/v took 36, 12 and 1 minute(s) respectively, to reduce the viable count of about 10^6 organisms ml^{-1} of Ps. aeruginosa by 5 log cycles; the times taken to reduce similar initial inoculum sizes of Staph. aureus by 5 log cycles were 24, 19 and 4 hours respectively at 25°C. The inactivating medium they used was that of Norton, Davies, Richardson, Meakin and Keall (53). Anderson, Lillie and Crompton (54) found that in 69 out of 75 eye-drops they tested, chlorhexidine (0.005-0.01%), was an efficient bacteriostatic preservative when challenged with low numbers of Staph. aureus, P. vulgaris and Ps. aeruginosa. Unfortunately, to reduce swarming in the nutrient agar plates used to incubate membrane filters carrying P. vulgaris, they included 0.1% phenol in the agar, making the results for this organism meaningless.

Chlorhexidine has several disadvantages:-

1) due to its cationic nature, it is incompatible with soap and other anionic material (51),
2) at 0.05%, it will precipitate most bicarbonates, borates, phosphates and sulphates (55); at 0.005%, however, no precipitation was observed in ophthalmic preparations containing this preservative (55),
3) its bactericidal activity is seriously reduced in the presence of organic material such as serum (54),
4) contamination of chlorhexidine solutions with Pseudomonas species has been observed (56, 57).

Polymxyin B Sulphate. This antibiotic has been recommended by the United States Pharmacopoeia (U.S.P.), to be used with benzalkonium chloride (26). Riegelman et al (35) found that 1000 units ml^{-1} of

polymxyin B sulphate killed 10^8 cells ml^{-1} of Ps. aeruginosa within 30 minutes. They used 0.5% lecithin in heart infusion agar as their inactivator. The activity of polymxyin B sulphate has been shown to be enhanced by EDTA (58).

Organic Mercurials. The most commonly used organic mercurial preservatives are phenylmercuric nitrate (PMN), phenylmercuric acetate (PMA) and thiomersal. PMN has the advantage over some organic mercurials in not being precipitated at a slightly acid pH. Brown (59) studied the activity of 0.002% and 0.004% PMN in both fluorescein and water against 24-hour cultures of 3 strains of Ps. aeruginosa, diluted to contain 10^6 cells ml^{-1}. He used the recovery medium of Kohn et al (15) and carried out his experiments at 22^oC. With PMN and fluorescein, he observed that concentration had only a marginal effect on activity and that kill times of under 3 and 5 hours respectively, were obtained. In the absence of fluorescein, kill times of between 5-24 hours and 3-5 hours respectively were found, depending on the concentration of PMN and the strain of Ps. aeruginosa used. Kohn et al (15) maintained that the antipseudomonal activity of PMN was too slow. They determined that both a 0.005% and a 0.01% solution took 6 hours to kill 10^8 organisms ml^{-1} of 24-hour cultures of 13 strains of Ps. aeruginosa, at 24^oC. The inactivating medium they found to be most effective contained a mixture of polysorbate 80 and lecithin in fluid thioglycollate medium.

Riegelman et al (35) showed that 0.01% PMN apparently produced sterility at the end of an hour's contact with a strain of Ps. aeruginosa isolated from an ocular infection, when subcultured into thioglycollate broth. They worked with a 24-hour culture and used an initial challenge level of 10^7-10^8 organisms ml^{-1}. Corneal ulcers in rabbits resulted from

these apparently sterile solutions though, even after a week's exposure to PMN. When the thioglycollate broth was replaced with a lecithin-polysorbate 80-thioglycollate medium, the discrepancy between in-vitro and in-vivo results was eliminated, with sterilising times being in excess of a week. They concluded that bacteriostatic concentrations of a preservative were inadequate for the preservation of ophthalmic products. Lawrence (9) found that organic mercurials were less effective in the presence of the common ophthalmic drugs. In several ophthalmic solutions, 0.01% PMN was shown to kill 24-hour cultures of 4 strains of Ps. aeruginosa in times varying from under half to 24 hours, at 24-26°C. In the absence of any drug, the identical concentration of PMN only required times of up to 3 hours. The recovery medium he used was Brewer's fluid thioglycollate medium.

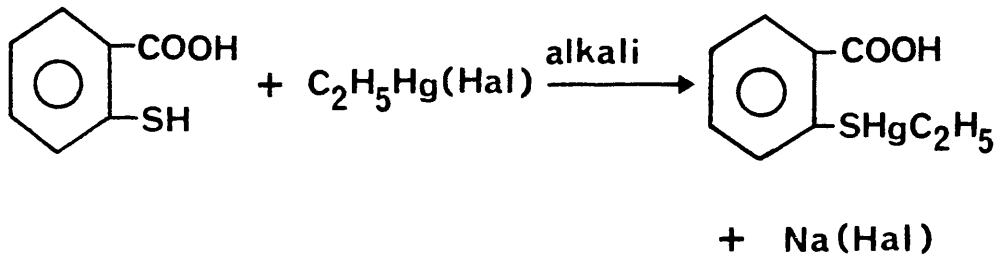
In 1963, Abrams observed that the long term instillation of miotic drops preserved with PMN (0.004%), could result in the appearance of mercurialentis, a yellowish-brown discolouration of the anterior lens capsule in a small proportion of the patients (60). It is difficult to draw any significant conclusions from these observations, however, due to the extreme variability of the occurrence between patients. Abrams, Davies and Klein (61) published their observations on patients who had been using miotics preserved with the other important organic mercurial, thiomersal, in 1965. The 21 cases of glaucoma they studied had received continuous therapy in the form of pilocarpine drops each day, over a period of 4-10 years. In none of these cases was there any convincing form of mercurialentis.

THE USE OF THIOMERSAL AS A PRESERVATIVE

Physical Properties of Thiomersal

Thiomersal, the sodium salt of ethyl mercurithiosalicylic acid,

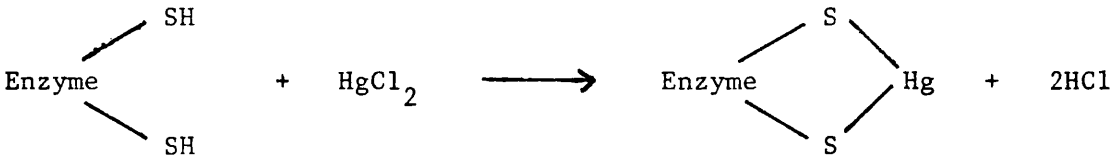
is a white crystalline solid, with a melting point at about 230°C. It is easily soluble in water and the lower alcohols, but insoluble in lipoid solvents (62); it is incompatible with acids, iodine, the heavy metal salts and many alkaloids (63). Thiomersal has a molecular weight of 404.8 and contains about 50% mercury. It is known by a wide variety of names which include Thimerosal, Thiomersalate, Thiomersalum, Merthiolate, Merzonin, Mertorgan and Merfamin. For the purposes of this thesis, it will be referred to as Thiomersal. Its preparation was patented by Kharasch in 1928 (64), and the reaction he used has been summarised below:-



Mode of Action of Thiomersal

One of the earliest ideas concerning the mechanism of action of mercurials is that they precipitate bacterial protein by combining directly with it to form a 'mercury-proteinate'. This theory arose because mercuric chloride was known to coagulate protein. In 1940, Fildes suggested that mercurials could act as inhibitory agents by interfering with an essential metabolite (65). Since their antibacterial action could be prevented/antagonised by hydrogen sulphide, an insoluble sulphide must be formed which removes any active mercury ions. As it is well known that R-SH compounds are essential metabolites in cellular

physiology, he suggested that the antibacterial action of mercury was due to interference with this essential R-SH grouping. Selzer and Baumberger had found in 1942, that finely divided mercury had an inhibitory effect on the respiration of yeasts (66). They assumed that this was due to an oxidation-complex formation between the sulphydryl groups of the enzymatic systems of the yeasts, and mercury. The oxygen uptake of Escherichia coli, Salmonella typhi and Staph. aureus has been shown to be completely inhibited by mercuric chloride (67). One of the constituent amino acids found in both structural and enzyme protein is cysteine ($SH.CH_2.CHNH_2.COOH$). The sulphydryl group derived from cysteine residues is essential for the activity of many important enzymes (68). Hugo in his review on the mode of action of antibacterial agents (5), suggests that many metals can react with sulphydryl enzymes to form mercaptides. He envisaged that the reaction with mercuric chloride would take the following form:-



This sulphydryl poisoning can be reversed in some cases by the addition of a sulphydryl compound to the reaction, and knowledge of this has been exploited in the development of inactivators for sterility testing.

Meyer in 1964, reported that mercurials acted mainly by physical adsorption and that lipid phases were somehow involved because most organic mercurials had an affinity for lipid (69). He stated that chemical binding with sulphydryl groups occurred on the surface, but that this was reversible. Irreversible, lethal effects occurred as a

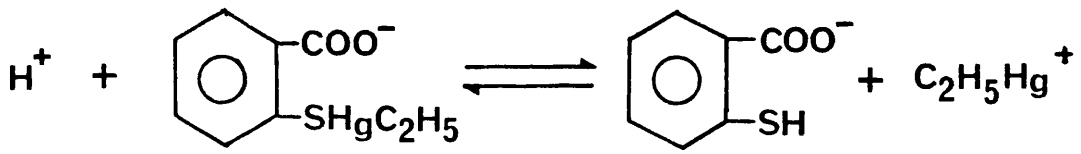
result of structural changes within the cell. This view was also held by Hess and Speiser (70), who envisaged that the action of mercurials probably proceeded in two stages, as outlined below:-

1) the mercurial is adsorbed on the cytoplasmic membrane, adsorption only being successful when charges of the interacting groups were opposite; the sulphydryl groups attacked in both Gram-positive and Gram-negative organisms were apparently the same; this first stage is essentially bacteriostatic and reversible by thioglycollate,
2) penetration through the lipid barrier then occurs, the water: lipid partition coefficient of the mercurial being the critical factor here; once it has penetrated, the action is no longer reversible and is therefore bactericidal.

The results they obtained apparently supported this theory, as phenylmercuric borate, which is easily soluble in lipids, was more active against the test organisms they used than thiomersal, which is poorly lipid soluble. The inoculum level they used was 3×10^5 organisms ml^{-1} from 24-hour cultures grown at $37^{\circ}C$, and their membrane filters were incubated on nutrient agar containing 0.05% thioglycollate as an inactivator.

Direct evidence for the mode of action of thiomersal is limited. It has been shown that a 0.0004% solution of thiomersal caused the lysis of some bacterial cultures (71). Low concentrations of thiomersal have also been demonstrated to cause the lysis of a growing E. coli culture, but not to affect isolated cell walls of this organism (72). This was postulated by the authors to mean that the former occurred as a result of interference with disulphide bond formation in the developing cell walls. Gould and Inglima (73) felt that the mode of action of

thiomersal was through 'the sustained release of mercurial ions which penetrate the cell and combine with various respiratory enzymes, producing metabolic inhibition'. The most detailed concept of the mode of action of thiomersal had been put forward in 1956 by Davisson, Powell, MacFarlane, Hodgson, Stone and Culbertson (74). They attributed its activity to a partial ionisation to give a low, but effective, level of the ethyl mercuric ion, $C_2H_5Hg^+$, as represented by the following equilibrium reaction:-



They suggested that the ethyl mercuric ion then blocked enzymatic processes by combining with sulphydryl groups on enzymes, and concluded that the value of thiomersal as a preservative, lay in the fact that it provided a reservoir of $C_2H_5Hg^+$, at low concentration.

Antimicrobial Properties of Thiomersal

In the 1930's, several laboratory studies were carried out on thiomersal (75-81). Powell and Jamieson were responsible for the first detailed investigations into the properties, activity and safety of thiomersal. They also presented data which showed its advantages as a preservative for vaccines, serum, toxins and anti-toxins. Unfortunately, none of these early studies used an inactivating agent for thiomersal,

so that the data from them is of historical value only. This also applies to much of the work carried out on thiomersal over the next twenty years (82-84).

Christensen carried out a comparative evaluation of the antibacterial activities of benzethonium chloride (phemerol) and thiomersal in 1963, using concentrations of the latter of 2.5×10^{-4}M and 6.2×10^{-5}M (85). He intended using a wide range of Gram-positive and Gram-negative organisms isolated from sterility tests as his challenge organisms. Fluid thioglycollate medium U.S.P. was used for his tube tests, but Christensen did not use an inactivator for his viable count determinations. Instead, he calculated the concentration of thiomersal that would have been carried over into the agar and then determined whether this would affect the growth of any surviving organisms. He established that Staph. aureus and B. cereus would be inhibited by concentrations of thiomersal less than or equal to, the residual thiomersal concentration transferred from the test medium and concluded that he was unable to use these organisms. With Ps. aeruginosa, however, he found that the minimum bacteriostatic concentration of thiomersal was more than 2.5×10^{-5}M, which was much higher than the maximum residual concentration of thiomersal that would be carried over after an initial 1:150 dilution of the preservative. On the basis of this finding, he evaluated the activity of thiomersal at 20°C, on 24-hour cultures of 11 strains of Ps. aeruginosa. The cultures were diluted before use, so that the initial challenge level was about a few thousand organisms ml^{-1}. The 2.5×10^{-4}M thiomersal solution was found to kill all 11 strains tested within 48 hours.

An interesting outcome of his work was the demonstration of a

synergistic activity between phemerol (5.4×10^{-5}M) and thiomersal (2.5×10^{-4}M). He showed that a strain of Ps. aeruginosa which was partially resistant to either preservative separately, was killed by a combination of both compounds within 24 hours at 20°C. The recovery medium he used here was fluid thioglycollate. The phenomenon of synergy between mercurials and surface-active compounds has been reported elsewhere (86, 87). Christensen was optimistic about the antibacterial properties of thiomersal as he had found it to be active at concentrations well below those normally used. He had also observed that contamination of preparations preserved with thiomersal only occurred very rarely, and that no clinical infection had ever been traced to contamination of such products (88).

Hugo and Foster (40) investigated the ability of eight bactericides to kill Ps. aeruginosa under experimental conditions related to the problems of achieving sterility in eye-drops. They looked at the concentrations of thiomersal required to reduce viable counts of 10 and 100 organisms ml^{-1} to zero in 30 minutes. Control experiments had shown that the media they used was capable of inactivating thiomersal. The temperatures they chose to work at were refrigerator temperature (4°C) and room temperatures in temperate climates (18°C) and the tropics (30°C). At 4°, 18° and 30°C respectively, 0.00175%, 0.001% and 0.001% w/v thiomersal respectively, were required to reduce an initial viable count of 10 organisms ml^{-1} to zero in 30 minutes. At the same temperatures, 0.0025%, 0.002% and 0.0015% w/v respectively were required to reduce an initial viable count of 100 organisms ml^{-1} to zero in 30 minutes. The authors also showed that 0.01% w/v thiomersal only required 10 minutes at 18°C, to kill 100 organisms ml^{-1} of the identical Ps. aeruginosa strain. Their results do indeed demonstrate that thiomersal

can be effective against Ps. aeruginosa, but the low inoculum levels they used, whilst relevant to 'in-use' situations, give minimal data about the kinetics of death. When larger challenge inocula are used, it is generally found that the kill times reported are considerably increased, and this has led to the use of thiomersal in ophthalmic preparations being questioned (15, 35, 89). One such example is provided by the data of Kohn et al (15), who found that 0.01% and 0.02% thiomersal required 9 and 6 hours respectively, to kill 10^8 organisms ml^{-1} of a 24-hour culture of Ps. aeruginosa at $24^{o}C$. The inactivating medium they used was fluid thioglycollate containing polysorbate 80 and lecithin.

Factors Influencing the Activity of Thiomersal

The Incorporation of EDTA in Formulations. Thiomersal is frequently formulated with the chelating agent, EDTA, but the role of EDTA in this context is rather confused. EDTA has been shown to enhance the activity of several preservatives, for example, benzalkonium chloride, against Ps. aeruginosa (43, 47, 58, 90, 91). Brown and Richards found that magnesium and calcium would block the potentiating action of EDTA (58). In the absence of EDTA, however, they noticed that these ions would antagonise the action of their preservatives. On the other hand, as these ions will chelate with EDTA, they probably prevent it chelating with ions in the bacterial cell membrane. They concluded that their findings were consistent with the hypothesis that 'EDTA exerts a lytic effect and is synergistic with antibacterial agents by a mechanism involving removal of calcium or magnesium ions or both, from the cell membrane'. As the structural integrity of the cell is affected, concentrations of the antibacterial agent previously unable to reach its site(s) of activity, are able to do so. Magnesium has been established as an

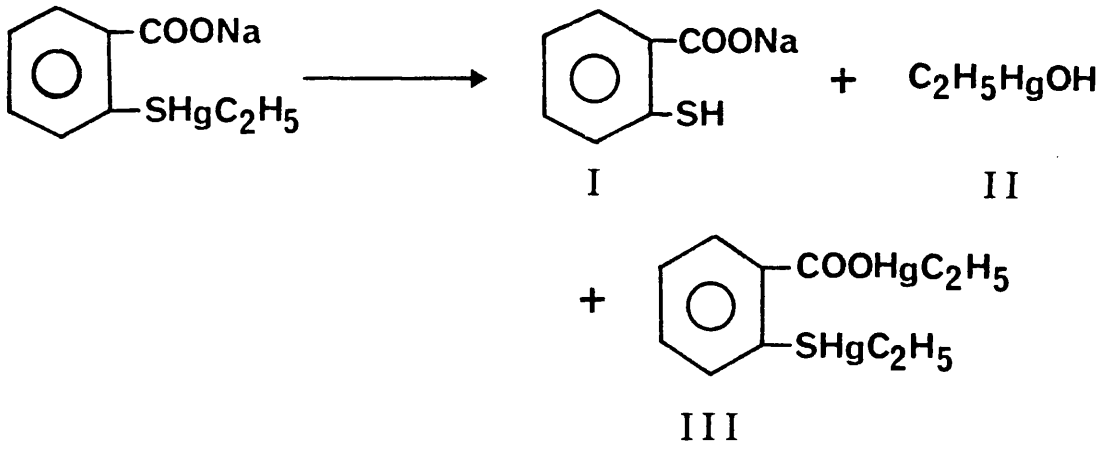
important stabiliser of ribonucleoprotein in bacterial cells (92-94), so that the chelating ability of EDTA could influence ribosome structure (95).

Richards and Reary (91) examined the effect of autoclaving thiomersal with certain adjuvants, and observed that EDTA-thiomersal combinations were less effective than thiomersal on its own. They used thioglycollate medium U.S.P. as an inactivator, and found that 0.01% w/v thiomersal killed an overnight, diluted culture of Ps. aeruginosa (3×10^5 organisms ml^{-1}), in 3.5-4.0 hours at 25°C. The identical concentration of thiomersal took longer than 6 hours to kill an overnight, diluted culture of Staph. aureus (8×10^5 organisms ml^{-1}) at 25°C. EDTA on its own at a concentration of 0.1% w/v, had no effect on either organism within 6 hours. When EDTA at 0.1% w/v was added to 0.01% w/v thiomersal, the time taken to kill the same inoculum size of Ps. aeruginosa was prolonged by at least 2 hours; the time taken to kill Staph. aureus was still in excess of 6 hours. The authors state that such inactivation of thiomersal by EDTA was contrary to the results obtained by Gould and Inglima (73) and Bixler (96). The former have shown that of the commercially available contact lens solutions they had examined, the most active products also contained EDTA. Only one of these was a thiomersal (0.004%) and EDTA (0.1%) combination though and as these results were obtained without the use of an inactivator, they are of little value.

Richards and Reary (91) autoclaved their thiomersal solutions, both with and without EDTA, for 20 minutes at 121°C, before their challenge tests. Although the B.P.C. does suggest that thiomersal may be sterilised by autoclaving, the authors did not include a control nor investigate if the inclusion of EDTA had any effect during the autoclaving process. Moreover, the pH of their two solutions is quite different, being pH 6.1 for thiomersal alone after autoclaving, and pH 4.95 for thiomersal with

EDTA after autoclaving. This difference in pH could markedly affect the stability of thiomersal, its subsequent interaction with EDTA, and hence the activity of the final product, and should, therefore, have been considered. The effect of acid conditions on the test bacteria has also been neglected.

The Effect of Light. In 1951, Tanaka and Mitsuno (97) reported that when exposed to sunlight, thiomersal would degrade to sodium thiobenzoate (I), ethylmercuric hydroxide (II) and the double mercury ester, ethylmercuri-2-ethylthiomercuri-benzoate (III), as shown below:-



Research carried out in this School has confirmed the presence of I and III in photodegraded solutions of thiomersal, by chromatography and mass spectrometry (98).

The photodegradation products of thiomersal were also studied by Ludtke, Darsow and Pohloudek-Fabini (99) and Tsuji, Yamawaki and Miyazaki (100). Tsuji et al looked extensively at the way different types of light could

affect the stability of thiomersal. They examined the effect of a fluorescent tube (20 W), a mercury lamp (300 W) and daylight in Japan (in August), on a fine day, a slightly cloudy day and a cloudy day, on the degradation of thiomersal (50 μg ml^{-1}) in phosphate buffer at pH 6. The residual thiomersal concentration was found to be lowest after an hour of sunlight on a fine day (39.0%), and highest after an hour of natural light on a cloudy day (88.5%). Their studies also showed that photochemical degradation was unaffected by metal ions or EDTA. Meakin and Khammas had also found that 0.1% EDTA did not improve the photostability of thiomersal (98). Tsuji et al (100) demonstrated that the photochemical degradation of thiomersal was affected by pH. When exposed to a mercury lamp for one hour, solutions of thiomersal (50 μg ml^{-1}) in buffer were found to be more stable between pH 5 and pH 7. Khammas showed that in citrate buffer, the reaction rate increased as the pH decreased from 6.5 to 5.0 (101). He also showed that the photochemical degradation of thiomersal was concentration dependent, the half life increasing with an increase in concentration.

The Effect of Heat. Tsuji et al (100) found that aqueous solutions of thiomersal were quite stable to heat, although no mention is made of the range of temperatures they looked at. When kept at 50°C over a 60-day period, alkaline solutions of thiomersal were found to be more stable than acidic solutions. Thermal decomposition was apparently accelerated by Cu^{2+}, Fe^{3+}, Zn^{2+} and Pb^{2+} ions, but not by Ca^{2+} or Mg^{2+} ions. Ludtke et al (99) looked at the percentage decomposition of thiomersal at 50° and 60°C over a period of 59 days, and found that 43% and 35% respectively, remained at the end of the observation period.

Interaction with Plastic Containers and Soft Contact Lenses. The work of Richardson, Davies, Meakin and Norton (102) has shown that thiomersal may be sorbed by polyethylene and polypropylene containers to such an extent that there is almost complete loss of the preservative on storage. This loss is not readily explicable as thiomersal is non-volatile, and no interaction would be anticipated between a carboxylate anion and a polymeric hydrocarbon. However, if degradation had occurred, and a mixture of ethylmercuric hydroxide and di(ethyl mercuric) thiosalicylate been formed, both of these could partition into a hydrocarbon polymer. The ethylmercuric hydroxide, being volatile, would then be lost from the container into the atmosphere. The presence of a filler such as titanium dioxide which is incorporated into commercial polyethylene containers, would result in a much more open network of polymer chains. These could then allow a greater and more rapid penetration of the degraded products. Where standard low density polyethylene containers were used, loss of thiomersal occurred at a much slower rate. Richardson et al (102) also investigated the interaction of thiomersal with polyhydroxyethylmethacrylate (polyHEMA), the major constituent of soft contact lenses. Of the four standard preservatives usually incorporated in contact lens solutions, only thiomersal in simple aqueous solution, did not interact with polyHEMA powder over a 24-hour period. The influence of pH on the binding of thiomersal at 30°C was also investigated. Above pH 5.0, binding was low, a maximum being reached at about pH 3.0. No uptake was found until some thiomersal was present in the un-ionised form, i.e., below pH 5.0. With commercial solutions formulated above pH 5 therefore, binding would not be a problem. However, below pH 5.0, an interaction does occur under laboratory conditions, and solutions formulated at these pHs', may bind thiomersal to soft lenses during soaking and cleaning operations

and subsequently release it into the eye.

Incompatibility with Rubber and Gum. The interference of rubber with the action of thiomersal has been shown (100, 103, 104). Different rubber products have been shown to be able to remove thiomersal from solution, and rubber caps pre-treated with thiomersal and placed in contact with a solution of thiomersal, were apparently as capable as untreated rubber caps of removing thiomersal from solution (103). Tsuji et al (100) had also found that thiomersal (50 μg ml^{-1}) in phosphate buffer at pH 7, was absorbed by a fragment of a rubber cap at temperatures ranging from 0-50°C. The extract from rubber caps boiled in distilled water was also found to accelerate the photodegradation of thiomersal solutions. The vulcanising compound, 2-mercaptobenzothiazole, was suggested to be one of the possible causes of this accelerated breakdown.

Toxicity and Sensitivity Studies on Thiomersal

In 1975, Blair, Clark, Clarke and Wood reported that monkeys that had been dosed intranasally each day over a six month period with 0.002% w/v thiomersal, had detectable levels of mercury in their tissues (105). 37-91% of the mercury was present in the inorganic form. The work of Clarkson (106), provides further evidence for the conversion of thiomersal to inorganic mercurial compounds, when he was able to show that inorganic mercury in proportion to total mercury ranging from 12-69%, could be detected in the tissues of a person who had been exposed to thiomersal for three months. The findings of these workers is in direct contrast to the view held by Bailey (107), that 'thiomersal breaks down to an organic mercury compound that is stable and readily excreted from the body and consequently does not lead to an accumulation of mercury in

the tissues'. No evidence for this statement was provided.

Topically administered medicaments can be a cause of allergic contact dermatitis. Hypersensitivity reactions often accompanied with erythema and papular or vesicular eruptions have been detected. Epstein reported on allergy to a range of topically administered medicaments that he had encountered over a three year period (108). He found that 6 of his 78 patients were allergic to 0.1% thiomersal. Reisman documents cases of delayed hypersensitivity to 0.1% thiomersal, when it had been incorporated in allergen extracts and diluent in use at an allergy clinic (109). He cites studies (110, 111), which suggest that either the mercurial component or the thiosalicylate component could be the antigen responsible for eliciting the delayed hypersensitivity reaction.

ASSESSMENT OF PRESERVATIVE ACTIVITY

Many contrasting reports have appeared in the literature on the efficiency of antimicrobial agents, differences which probably reflect the varying experimental procedures in use in different laboratories. Despite this, however, official compendia and national regulatory bodies are apparently reluctant to specify an internationally standardised procedure. Without question, the only logical approach to preservative evaluation must be based on the concept of 'challenge tests'. This phrase has unfortunately been interpreted in different ways, and may range from tests which constitute a bare minimum of challenges, through to tests which are needlessly elaborate. Often, the factors constituting a realistic testing procedure are clouded by a lack of specific detail, so that very little or very questionable data is produced. When one considers that product safety and integrity is based on these results, great care must be exercised in selecting relevant methods of evaluation.

Methods of Assessing Antimicrobial Activity

An extensive review of the methods of testing preservative efficiency has been compiled by Cowen and Steiger (112). Generally, the evaluation of antimicrobial activity can be determined by methods which fall into four groups (113):-

1) end-point methods,

2) death rate studies, based on viable count determinations,

3) photometric methods,

4) biochemical methods.

End-point or Extinction Methods. These are essentially tests for sterility, carried out after treatment of bacterial suspensions with the antibacterial agent for specific times and under controlled conditions. They differ in the manner in which the experimental findings are calculated, as well as in details of experimental procedure.

Phenol Coefficient Tests. The two official British methods are the Rideal-Walker (R-W) test (114) and the Chick-Martin test (115). The methods of the Food and Drug Administration (F.D.A.), and the Association of Official Agricultural Chemists (A.O.A.C.) are used in the United States (116, 117). Essentially, these tests follow the same basic principles, namely, phenol is used as the reference standard; the test solutions are simple dilutions in water, with the exception of the Chick-Martin test which uses yeast as organic matter; the killing time is short (minutes); the end point is that of a virtual kill, and activity is expressed as a coefficient related to the lethal effect of phenol.

The R-W test was the first phenol coefficient test to be devised. Originally published in 1903, it has since undergone several modifications

leading to its present form. Serial dilutions of disinfectant and phenol at 17-18°C, are inoculated with a defined volume of bacterial culture and subcultures made into nutrient broth at 2.5 minute intervals, up to 10 minutes. The tubes are then incubated for a fixed time and the phenol coefficient calculated by dividing the dilution of disinfectant that killed in 7.5 minutes, but not in 5 minutes, by the dilution of phenol that showed the same end point. In the F.D.A. method, the end point is the lowest concentration of disinfectant and phenol killing in 10, but not 5 minutes, at 20°C. The A.O.A.C. method differs from the F.D.A. method in that a number of subculture media may be used, depending on the test disinfectant.

These tests can all be criticised for extrapolating data obtained from them to other situations and disinfectants very different from phenol. As Berry pointed out (118), all phenol coefficient tests contravene the fundamental rule of biology, that like should be compared with like.

Determination of the Mean Death Time or Mean Single Survivor Time. In 1954, Berry and Bean proposed a method which relied upon the estimation of bactericidal activity from extinction time data (119). The test has the advantage in that the entire reaction mixture is sampled, thus eliminating the errors normally inherent with sampling. Despite this, however, it has never been widely used.

The Kelsey-Sykes Capacity Test. This test has its origins in 1965, when Kelsey, Beeby and Whitehouse published details of a test for disinfectants (120). The Kelsey-Sykes test (121), published in 1969, envisaged two conditions of use, namely, 'clean' and 'dirty', to represent the absence and presence of organic matter. Whilst this test was devised primarily for hospitals, the authors felt that it could easily be adapted for other situations. An improved version of the Kelsey-Sykes capacity test (122) was issued in 1974. The main features of this have been

summarised below:-

1) all dilutions of the disinfectant are carried out in hard water prepared as recommended by the World Health Organisation,
2) a chemically defined liquid medium is used for growing the test organisms,
3) a watery suspension of the test organism is used in the test under 'clean' conditions,
4) a yeast-test organism suspension is used for the test under 'dirty' conditions,
5) a nutrient broth containing 3% w/v Tween 80, is used as recovery for all disinfectants.

This improved test, besides making the interpretation of results much simpler, illustrates how a test can be designed to standardise such important parameters as the choice of test organism, growth conditions, experimental conditions, including the holding medium, temperature and pH, and recovery conditions.

Viable Count Determinations. There is no doubt that the maximum information concerning the fate of a bacterial population is gained by performing viable counts at pre-determined time intervals. The number of survivors expressed as a percentage remaining viable at specified times can be calculated, and used in assessing activity. Viable counting is based on the assumption that each countable colony arises from a single viable cell in the original sample. Because of the need to use colony formation as a test, the viability of an individual microbe can only be determined retrospectively. As soon as its viability is established, it ceases to be the same individual. It is also possible under certain conditions, to obtain microbes with many of the characters of normal living organisms, but which are unable to divide

and form a colony (123). A major source of error can also arise from the clumping of the test organism, so that one colony in the final plate may arise, not from one organism, but from several. Despite these limitations, however, viable counting remains the most popular method of assessing antimicrobial activity.

Photometric Methods. Measurements of the light scattering of bacterial cultures are measurements of the total numbers of bacteria present within certain limits, and the optical density reading at a given time is a function of the inoculum size, bearing these limits in mind. These methods rely on treating an identical series of cultures with differing concentrations of a disinfectant for a fixed time, and then removing a standard volume of the treated culture and using this to initiate a fresh culture. The level of growth achieved after incubation for the same time, is then taken to reflect the degree of kill originally achieved. The application of these principles to the evaluation of disinfectants has been discussed (124), and although it has been used in this context (125), the method has never had a widespread popularity.

Biochemical Methods. These rely on an investigation into the inhibition of some easily followed metabolic process, such as, the uptake of oxygen by bacteria and yeasts (66, 67, 126), the evolution of carbon dioxide from sucrose by yeasts (127), or the reduction of methylene blue by bacteria (128). The success of these methods lies in the close relationship between the inhibition of the metabolic process and the death of the cell. The evidence available indicates that in most cases, the magnitude of the effect may not parallel cell viability (123, 126), and for this reason, these methods are not favoured for the evaluation of antimicrobial activity.

Effects of Pre-treatments

Choice of Test Organisms. The initial phase of a meaningful preservative evaluation rests on the selection of appropriate challenge organisms. Two schools of thought govern this choice, namely that:-

1) test organisms should be selected laboratory strains available from culture collections, or
2) test organisms should be selected from the environment or from contaminated products.

Subscribers to the latter point of view believe that the use of standardised laboratory organisms with a 'pedigree', may result in a false sense of complacency about the adequacy of preservation of a product.

The U.S.P. XX recommends the following five organisms, namely, C. albicans (ATCC 10231), A. niger (ATCC 16404), E. coli (ATCC 8739), Ps. aeruginosa (ATCC 9027) and Staph. aureus (ATCC 6538). In addition, the U.S.P. states that other organisms may be included in the test, especially if they represent contaminants likely to be introduced during use of the product. The B.P. 1980 recommends the same organisms, but omits E. coli and suggests a different strain of Ps. aeruginosa (ATCC 19429). The B.P. also qualify their specification by suggesting that other strains or species likely to be found in conditions under which a product is made or used, or which might offer a specific challenge to the type of product tested, may be included.

Yablonski (13) felt that in addition to certain culture collections, the use of micro-organisms isolated from contaminated products with formulations similar to the test product, would mean challenging with organisms that had the greatest ability to survive the product environment. Moore (129), whilst agreeing with Yablonski, felt that the list should also include organisms recently isolated from nosocomial infections of the human body with which the preparation was likely to come into contact.

Davies (37) felt that the use of many different test strains was unnecessary, as the relationship between their 'in-vitro' and 'in-vivo' behaviour is a tenuous one. Moreover, as the predominating contaminant flora has been shown to vary from region to region (33), inter-laboratory comparisons are difficult unless standardised challenge organisms are included in a test series.

Growth Media. There have been many developments in recent years in the importance of the role played by the bacterial envelope in resistance to antibacterial agents and antibiotics. In particular, it has been established that when faced with the lack of an essential nutrient, a dividing bacterium will make an envelope characteristic of the particular depletion (130). The effect of different nutrient depletion, besides altering the general biochemistry of the cell, will also result in cultures with radically different envelopes (131, 132). The phenomenon was found to be particularly noticeable with regard to the outer membrane of Gram-negative bacteria (133, 134), but Gram-positive bacteria (135, 136), and yeasts (137), have all been reported to vary in sensitivity to antimicrobial agents with conditions of vegetative growth. The implications of this for any Pharmacopoeia tests for preservative activity are apparent (130, 138).

Commercially available media contain a wide variety of components which would be impractical to standardise, and batch to batch variations are inherent to the use of such media. Moreover, when autoclaving is used as a method of sterilisation, these separate components could undergo different degrees of degradation (139). The use of chemically defined media sterilised by membrane filtration, would seem to be the best solution to this problem, as, whilst the filtration of liquid media may also lead to changes in composition affecting the biological value of the sterile filtrate, in practice, these effects are only very

slight (139).

A strain of Ps. aeruginosa grown in inorganic salts solution (140), was shown to be more resistant to the effects of sonication, ultra-violet radiation, salt (3.5% sodium chloride solution) and temperature (65°C). Brown pointed out, however, that a conceptual error can arise from believing that a chemically defined medium necessarily defines a bacterial culture, as often the nature of a growth-limiting nutrient is unknown (130). Nevertheless, the use of a chemically defined medium does enable standardisation to be achieved. This is illustrated by the results of Al-Hiti and Gilbert (141), who concluded that the use of different growth media could be a primary cause of inter-laboratory variation. The U.S.P. antimicrobial effectiveness test does not specify precise growth conditions, and the lack of these could influence the results of challenge tests, especially when the concentrations of preservatives used are barely adequate. Whilst the authors felt that there was indeed justification for designating a single medium for the growth of each test organism, they were unable to decide whether this medium should be chemically defined and of low complexity, or undefined. They pointed out that the major advantage of chemically defined media is that these would reduce variation greatly and so increase test reproducibility.

Age of Test Culture. It is well known that the composition of a bacterial cell wall varies with the age of the culture. Different metabolic states have been postulated for different phases of the individual growth cycle, which result in cells in the middle region of the cycle being the most resistant, and those in the early and late phases being the most susceptible to damaging effects by chemical or physical agents (142). It has been demonstrated that exponentially

grown Aerobacter aerogenes were more sensitive than stationary phase A. aerogenes to starvation in saline phosphate buffer (pH 6.5), at 37°C (143). Carson, Favero, Bond and Petersen (144) showed that the phase of growth of a naturally occurring strain of Ps. aeruginosa affected its sensitivity to disinfectants. The reason for this change in sensitivity is not known. Some factors that have been shown to vary with age are:-

1) the teichoic acid content of Gram-positive cell walls,

2) the size of cells and thickness of cell walls,

3) the lipid content of cytoplasmic membranes.

Teichoic Acid Content. This has been demonstrated to be greater in mid-logarithmic phase cultures than in stationary phase cultures (145,146). The teichoic acids are responsible for the structural integrity of a wide variety of Gram-positive organisms and have also been suggested to function in the processes of cation assimilation (131).

Size of Cells. Cells from exponential phase cultures (2-9 hours at 37°C), were found to be much larger in length and width than cells from stationary phase cultures (24 hours at 37°C) (147). In addition, cell walls of Clostridium welchii have been reported to be thicker in stationary phase than in exponential phase (148).

Lipid Content. Studies on the cytoplasmic membrane of Streptococcus faecalis (149, 150), showed that the total lipid content was affected by the growth phase, being higher in stationary phase (40%), than in exponential phase (28%). In view of the involvement that lipid has been shown to have on the acquisition of resistance to antibacterial agents (5), these findings may have some relevance.

Effect of Inoculum Size. Cell concentration has been shown to affect the heat resistance of non-sporing micro-organisms, dense populations

being the more resistant (151). At 40°C, the death rate of A. aerogenes in phosphate buffer (pH 7.1) was shown to be slower, as the population increased up to 10^8 organisms ml^{-1} (152). The work of Postgate and Hunter (153) confirmed that dense populations of A. aerogenes survived longer than sparse ones.

The U.S.P. XX recommends that the concentration in the test preparation immediately after inoculation be between 10^5-10^6 organisms ml^{-1}; the B.P. 1980 suggests that the final concentration should be about 10^6 organisms ml^{-1}. Yablonski felt that the level to be used must depend on the degree of confidence desired by the manufacturer who might wish to assure himself that his product was capable of resisting microbial contamination, under both normal and abnormal conditions of product use and abuse (13). Davies, Richardson and Anthony (154) carried out an investigation into the influence of three inoculum sizes, namely, 10^6, 10^4 and 10^2 organisms ml^{-1}, on the antibacterial activity of the four common ophthalmic preservatives. Their results showed that benzalkonium chloride and thiomersal appeared to be affected most by the initial inoculum sizes of Ps. aeruginosa and Staph. aureus. With thiomersal at 0.004% w/v, the times taken to reduce the viable count by three log cycles were 7, 5 and 3 hours respectively for Ps. aeruginosa, and 32, 20 and 8 hours respectively for Staph. aureus.

Effect of Growth Temperature. All bacteria have an optimum temperature for growth, although they will tolerate a range of growth temperatures. Whilst growth temperatures have been shown to affect the subsequent sensitivity of micro-organisms, work in this field has been mainly concerned with survival studies and physical processes, such as the effect of heat. In this context, it has been shown that the heat resistance of many bacteria and yeasts increases with an increase in growth

temperature (151).

The effect of growth temperature on the assessment of antimicrobial activity is less clear. The work of Carson et al (144) did show, however, that growth temperature affected the subsequent susceptibility of a strain of Ps. aeruginosa to disinfectants. Young cells of E. coli from cultures growing slowly were apparently more resistant to several deletrious factors than were cells from rapidly growing cultures (155).

Effect of Harvesting Techniques and Diluents. The method of preparing a test inoculum has been a source of much debate. The U.S.P. XX suggests using sterile saline TS to wash surface growth into a suitable vessel, whilst the B.P. 1980 recommends using 0.1% peptone for the same purpose. Norton et al (53) filtered 1ml of a 24-hour culture of each of their test organisms through a membrane filter and washed with a minimal salts medium. Richards felt that as far as Ps. aeruginosa was concerned, this could affect its subsequent resistance to antimicrobial agents (156). He compared the results he had obtained using broth suspensions of Ps. aeruginosa with those of Norton et al (53), and in most cases showed that the former were more resistant to both commercial and laboratory-prepared contact lens solutions. Brown, in his study on the survival of Ps. aeruginosa in fluorescein solution (59), had also noted that broth exerted a protective effect. He found that water-washed cells lost their viability in fluorescein solutions in less than a day. In contrast, Cook and Wills (157) showed that suspensions of E. coli consisting of washed cells suspended in phosphate buffer (pH 7.0), maintained a higher viability and resistance to phenol than suspensions of unwashed, or washed cells suspended in water. They grew their test organism for 24 hours on a peptone agar slope and the initial inoculum size they obtained was about 2×10^9 organisms ml^{-1}. Davies and Norton

investigated the antipseudomonal activity of 0.004% benzalkonium chloride, using dilutions of an overnight culture in both a minimal salts medium and in Tryptone Soya broth (158). Their results show that there were only minor differences in kill attributable to the differences in these two harvesting systems, and concluded that the discrepancies reported by Richards must arise from some other factor(s). The importance of using an appropriate diluent cannot be over-emphasised. Jayne-Williams (159) stated that the function of a diluent was to enable a true assessment to be made of the status of a bacterial population; for this purpose, death or revival of the organism should not occur during the dilution process. It is, therefore, desirable, that the diluent should resemble the environment from which the micro-organisms concerned are derived. Suspension in distilled water has been shown to cause the death of certain bacteria (160, 161). Smith and Wyss (162) showed that with a 60-minute exposure, 10^{-2}M and 10^{-3}M phosphate buffer (pH 7.4) were optimum for preserving the viability of 2×10^{3} cells ml^{-1} of a culture of Azotobacter, that had no viable organisms after an equivalent time in distilled water. Straka and Stokes (163) found that rapid and extensive destruction of the bacteria isolated from poultry pies occurred in distilled and tap water, physiological saline and phosphate buffer at pH 7.2. The use of 0.1% peptone provided full protection for at least an hour. This result was supported by the findings of King and Hurst who showed that 0.1% w/v peptone water was the best diluent for Staph. aureus, Strep. pyogenes, E. coli and S. typhimurium (160). The major drawback to the use of peptone water is that it permits limited metabolism to occur.

Low concentrations of Mg^{2+} have been shown to prolong the survival of both Gram-positive and Gram-negative 'starved' bacteria (151, 164). The role of Mg^{2+} has been attributed to be at least partly due to the

stabilising effect it has on bacterial ribosomes (92, 93), as well as its potential action in reducing the toxic effects of certain metal cations (164, 165).

Factors which have a Bearing upon Experimental Conditions

Temperature. An increase in temperature is normally accompanied by an increase in the antimicrobial activity of a preservative and the effect of temperature on activity can be expressed as shown below (113):-

$$\theta^{(T_2 - T_1)^\circ} = \frac{k_2}{k_1} \qquad (3)$$

where θ is the temperature coefficient per degree rise, and k_1 and k_2 are the rate constants at temperatures T_1° and T_2° respectively. The rate constant is inversely proportional to the extinction time, where the extinction time is the time taken to sterilise the inoculum used. Providing the same inoculum level is used for the experiment carried out at T_1° as that used at T_2°, then,

$$\theta^{(T_2 - T_1)^\circ} = \frac{t_1}{t_2} \qquad (4)$$

where t_1 and t_2 are the extinction times at T_1° and T_2° respectively. The Q_{10} value (the coefficient per ten degree rise), can be calculated by determining the extinction time at two temperatures differing by exactly ten degrees, hence,

$$Q_{10} = \frac{\text{Time taken to kill at } T^\circ}{\text{Time taken to kill at } (T + 10)^\circ} \qquad (5)$$

High temperature coefficients are said to be characteristic of reactions involving denaturation or coagulation of protein (166). These are apparently particularly associated with oxidising reactions, whereas low coefficients are associated with reducing reactions. Usually, the

rise is a two or four-fold increase for each ten degree rise in temperature, but may be much higher for phenols. The actual values recorded appear to vary between laboratories and with organisms.
Occasionally, an increase in temperature can decrease antimicrobial activity. Davies (37) showed that the activity of chlorhexidine against Ps. aeruginosa was decreased about three-fold, when the temperature was increased from 15° to 25°C. In general, however, an increase in temperature is accompanied by an increase in antimicrobial activity (167).

pH. The effect of pH can be two-fold (168):-

1) an effect on the molecule of the antimicrobial agent,

2) an effect on the cell surface.

With some compounds, the active species is the unionised molecule, whilst the ion is inactive. Examples of these include benzoic acid, phenols, salicylic acid and acetic acid (113). Here, conditions of pH which favour the formation of the ions of these compounds will obviously result in a reduction in antimicrobial activity. In other cases, the activity of the drug is due to the ionised molecule, so that factors favouring ionisation enhance the antibacterial activity. Before an antibacterial agent can affect a cell, it must first 'combine' with it. It has been suggested that an increase in pH will increase the number of actively charged groups on the bacterial surface and, therefore, influence the number of positively charged molecules that can be attracted (169). The sulphydryl group of cysteine has a pK_a of 10.8 (170), so that the more alkaline the solution of cysteine, the greater its negative charge. For thiomersal, therefore, Hess and Speiser (171) felt that in more acid solutions, a greater number of the negatively charged thiomersal could be bound by sulphydryl groups. The results they obtained appeared to support this reasoning.

0.01% thiomersal in isotonic phosphate solution with potassium chloride was prepared at pH 4, 5.5, 7 and 8.5. 24-hour cultures of E. coli and Staph. aureus grown at 37°C, were diluted to contain 3 x 10^5 organisms ml^{-1}. The membrane filtration technique was used to assess viability and filters were incubated on nutrient agar supplemented with 0.05% thioglycollate. With E. coli, no survivors were detected at pH 4 or 5.5 after 24 hours contact at 20°C. With pH 7 and 8.5, the survivors were too numerous to be detected at the same time interval (in excess of 3000 colonies). With Staph. aureus, no survivors were detected at pH 4, 50-100 colonies were found at pH 5.5 and the filters were uncountable at pH 7 and 8.5 (colonies in excess of 4000). The effect of pH alone on the test organisms was not studied so that its contribution to the kill obtained is not known. Moreover, their reasoning does not acknowledge the effect of the $C_2H_5Hg^+$ ion, which one would anticipate being bound to a greater extent at alkaline pH.

Slightly acid conditions (pH 6.1-6.5) have been reported to favour the survival of A. aerogenes (150), whereas near neutral pH (pH 6.98) is apparently preferred by Strep. lactis (164). Anderson and Crompton (172) looked at the effect of pH on the survival of Ps. aeruginosa, as part of their study into the effect of varying pH on the antimicrobial activity of chlorbutol and benzalkonium chloride. They used citric acid/phosphate buffers at pH 3, 4, 6 and 7, a challenge inoculum of 10^4 organisms ml^{-1}, and membrane filtration as their method of assessing viability. After 20 minutes in the appropriate buffer at 20°C, the suspensions were filtered and the membrane filters incubated at 37°C for ten days. At this time, the pH 6 and 7 filters had confluent growth, the pH 4 filter showed a 99.8% kill and at pH 3, there was 100% kill. The strain of Ps. aeruginosa they used was thus shown to be very sensitive to acidic conditions.

El-Nakeeb and Farouk (167) also investigated the effect of pH on the antimicrobial activities of some preservatives. They used Sörensen's phosphate buffer (pH 5-8.5), and an inoculum level of 1.8×10^7 cells ml^{-1} of Staph. aureus. Their test solution contained 0.004 $\mu g\ ml^{-1}$ thiomersal in water and was sterilised by autoclaving. Turbidity readings were made after 24 hours at 37°C. Thiomersal was found to be insensitive to the range of pH tested and only about 5% growth inhibition was obtained. Once again, no controls were included, so that it is difficult to determine if this lack of effect is as a result of the test solutions having been affected in some way by the autoclaving process, or whether the effect is simply due to the limited range of pH tested. Moreover, no inactivators for thiomersal were included in their nutrient broth so that the data they obtained is of reduced value.

Tonicity. Lachrymal fluid is isotonic with blood and has a tonicity equivalent to that of 0.9% sodium chloride (173). It has been shown, however, that the eye can tolerate a range of tonicity from 0.5-2.0% sodium chloride equivalent (174). The tonicity of preservative solutions has been shown to markedly affect their activity. Davies et al (154) showed that the activity of thiomersal was greatest against Staph. aureus in isotonic solution, whereas that of benzalkonium chloride, chlorhexidine gluconate and chlorbutol was greatest in hypertonic solution. With Ps. aeruginosa, activities were increased two to three-fold in hypotonic solutions, apart from with chlorbutol.

Effect of Recovery Treatments

Inactivating Agents. It is now well recognised that all recovery media should routinely incorporate inactivating agents. These must themselves

be non-toxic to the test micro-organisms, and must inactivate the antimicrobial agent to give a product which is also non-toxic (168). Sulphydryl compounds have been used to inactivate inorganic and organic mercurial compounds since Fildes' discovery in 1940 (65), that mercury could combine with sulphydryl groups on enzymes. Sodium thioglycollate has been widely used as an inactivator for organic mercurials, although a report has been made that it markedly decreased the germination rate of spores of Cl. bifermentans, despite being necessary for the outgrowth of spores (175). It has been shown to be the most efficient mercurial antagonist, with cysteine being intermediate and glutathione being the least effective (176). It is thought that the poor activity of glutathione as a recovery agent is due to its being insoluble in lipid, and, therefore, being restricted to reacting only with free mercury present in solution or adsorbed on the bacterial cell. Sodium thioglycollate, however, being soluble in both the aqueous phase and to a limited extent in lipids, is able to penetrate the cell and, it is claimed, may antagonise any mercury within. A study of the factors influencing the stability of thioglycollate solutions has been made (177).

Richards and El Khouly investigated the effect of sodium thioglycollate on the recovery of E. coli, Ps. aeruginosa and Staph. aureus treated with PMN at 0.0025% and 0.00125% at 25°C (178). The test organisms were grown for 24 hours before being harvested and an initial inoculum size of about 10^9 cells ml^{-1} was used. Their results showed that the way in which thioglycollate was used influenced the data obtained. When it was included in the diluting fluid, it was more effective as an inactivator than when it was included in the nutrient agar. The optimal thioglycollate concentration for recovery was shown to vary with the test organism, being 0.4% for treated E. coli, 0.2% for Ps. aeruginosa and 2-3% for Staph. aureus. Elkhouly and Yousef (179) confirmed these

results with other mercurials, including thiomersal at a concentration of 0.05%.

For preservative testing, both the U.S.P. and the B.P. merely state that suitable inactivators should be included. Norton et al (53) used a general purpose recovery medium that consisted of a mixture of Tween 80 (3.0% w/v), 90% lecithin (0.2% w/v), sodium thioglycollate (0.1% w/v), Tryptone Soya broth (3.0% w/v) and distilled water (to 100%). This medium was shown to be able to support the growth of as low as 10 organisms, in the presence of the contact lens solutions they tested. The concentrations of thiomersal present here ranged from 0.001-0.004%, and at these levels, the thioglycollate concentration was apparently sufficient.

Composition of Plating Media and Effect of Envirionmental Conditions.

It has been stated that bacterial populations that have been partially damaged by physical or chemical agents, show different proportions of survivors according to the character of the medium used to assay viability (180). Rich media are reputed to give a better recovery than minimal media. Straka and Stokes (181) studied the effect of plating medium on the recovery of Ps. fluorescens (2×10^6 cells ml^{-1}), after freezing in 0.5% beef extract and storage at $-18^{\circ}C$ for one day. The injured cells were unable to grow on a simple glucose-salts agar medium, but could develop on the rich, complex medium, trypticase soy agar. They suggested that injury was probably being expressed as a nutritional requirement for materials that were peptide in nature. In contrast, Hess and Speiser (171) found that although enriched media promoted the rate of growth, they were unable to demonstrate any outstanding difference in the numbers of survivors obtained after treating E. coli and Staph. aureus with several antibacterial agents.

Jacobs and Harris studied the effect of environmental conditions and plating medium on the viability of bacteria damaged by phenols (182, 183). They found that damaged Staph. aureus and E. coli were very sensitive to their environment (182). Damaged cells inoculated into nutrient broth died unless Norit (0.1% w/v) was present; then, both the rate and extent of death was reduced. The addition of cations, especially Mg^{2+} (10^{-3}M), to broth, favoured the survival of damaged E. coli, but did not influence that of Staph. aureus. Jacobs and Harris also showed that bacteriological agar was toxic towards E. coli and Staph. aureus (183). When nutrient agar was treated with Norit (0.1% w/v), or ferric chloride (0.03% w/v), larger colonies were obtained from untreated suspensions of Staph. aureus; phenol-treated suspensions were found to give higher recoveries on both modified agar types. Magnesium (0.00185M) was again shown to be useful in inducing higher counts of damaged cells. Norit was suggested to function by removing toxic cations, such as those of heavy metals known to occur in commercial peptones.

The changes microbiological media can undergo during storage, such as the loss of moisture, can also be important (139). Agar plates will dry out at their surfaces and because water diffusion in agar gels may be a slow process, the surfaces may soon resemble the relative humidity of their environment. The prolonged storage of media will also increase the probability of contamination, which may remain undetected until incubation.

The temperature of incubation has been shown to affect the recovery of survivors. Jacobs and Harris (182) found that with untreated E. coli, incubation at 25°, 30°, 37°, 40°, or 44°C for 72 hours, had a marginal effect on the colony count, although there was a slight reduction at 44°C. Phenol-treated E. coli, however, gave noticeably lower counts

at 25°, 40° and particularly at 44°C, than those incubated at 30° and 37°C. Untreated Staph. aureus grew equally well at 37° and 40°C, and somewhat less well at 25° and 30°C; at 44°C, the colony count was often zero. With phenol-treated Staph. aureus, however, the best counts were obtained at 25° and 30°C; lower counts were found at 40°C and at 44°C very poor recovery was obtained.

MATERIALS AND METHODS

MATERIALS AND METHODS

General Methods

Glassware

Analytical Work. Glassware here was of grade A,'E-mil', Pyrex.

Other Work. Erlenmeyer flasks (Pyrex), narrow-necked and of capacity 250ml were used for the growth of micro-organisms. They were capped with 1" metal caps (Oxoid Ltd.). Two types of test tube were used:-

1) 150mm x 16mm, standard-walled, test tubes,

2) 150mm x 24mm, heavy-walled 'boiling' tubes.

Both types of tube were rimless, Pyrex, and capped with metal caps. The following types of bottles were also used:-

	Capacity (ml)
McCartney	25
Universal	30
'Medical Flat'	100
Duran	500 & 1000

1ml and 10ml colour snap, white, neutral glass ampoules DIN TYPE BS 795:1961 (FBG--Trident Ltd., London), were used where indicated. Spreaders were prepared from 10cm lengths of glass rod, 2mm in diameter. They were bent in a flame to an angle of about 60^{o}, to give about 30mm of spreading portion and a 70mm handle.

Cleaning of Glassware

Analytical Work. A chromic acid mixture was used to ensure thoroughly clean, grease-free glassware, by filling or immersing the vessels in the mixture for 30-60 minutes. This was followed by six rinses in running tap water and three in glass-distilled water.

Other Work. Visually clean glassware was cleaned with a dilute solution of LINKDET 708 (Link Chemicals Ltd.), and rinsed as above. Any soiled

glassware was soaked overnight in a 20% solution in distilled water of RBS 25 (Chemical Concentrates (RBS) Ltd.), before being subjected to the rinsing schedule.

Dry Heat Sterilisation of Glassware

Aluminium foil was used to envelop the openings of the larger items of glassware; glass pipettes were placed in aluminium pipette cans and spreaders and ampoules were placed in beakers covered with foil. All items were then placed in a Griffin 200 FC Oven (Gallenkamp & Co. Ltd.). The minimum holding time for sterilisation was 1 hour at 160°C.

Chemicals

All chemicals used were AnalaR grade and obtained from BDH Chemicals Ltd., with the following exceptions:-

di-Potassium hydrogen orthophosphate, anhydrous, SLR	Fisons Ltd.
Thiomersal, Laboratory Reagent Grade	BDH Ltd.
Sodium thioglycollate, Laboratory Reagent Grade	BDH Ltd.
EDTA, Laboratory Reagent Grade	BDH Ltd.
'Tween' 80, Laboratory Reagent Grade	BDH Ltd.
Lecithin, egg about 90% (stored at 4°C)	BDH Ltd.
N/1 Sulphuric Acid (VOLUCON)	Fisons Ltd.
N/1 Sodium Hydroxide (VOLUCON)	Fisons Ltd.
Hydrochloric Acid, SLR	Fisons Ltd.
Hexane, H.P.L.C. Grade	Fisons Ltd.
Propan-2-ol, H.P.L.C. Grade	Fisons Ltd.
95% Ethanol, SLR	Fisons Ltd.
Cetyltrimethylammonium bromide (re-crystallised before use)	BDH Ltd.

Acetonitrile, H.P.L.C. Grade	Rathburn Chemicals Ltd.
Methanol, H.P.L.C. Grade	Rathburn Chemicals Ltd.

All ten amino acids used in the defined media were chromatographically homogenous, and purchased from BDH Ltd., apart from L-Cysteine Hydrochloride, which was obtained from Hopkin & Williams Ltd. The vitamins used as media supplements were not less than 98% pure, and obtained from Sigma London Chemical Company Ltd., with the exception of nicotinic acid, which was a Nutritional Biochemicals Corporation product. Both biotin and pyridoxin were stored under desiccation, the former at 4°C, and the latter at -20°C. The combined amino acids media supplement used when indicated was BACTO Vitamin Assay Casamino Acids (Difco Laboratories, USA).

Diluents, Buffers and Test Solutions

Water. All water used throughout this work was glass-distilled. Its pH was found to vary between 4.7 and 5.5; for microbiological work, it was required sterile. All analytical work was carried out with double-distilled water, freshly obtained from a Fi-streem four litre, bi-distillation unit (Fisons Ltd.).

Davis - Mingioli Medium (DM). DM is the minimal salts medium of Davis and Mingioli (184). It was prepared as a double strength solution and diluted prior to use with an equal volume of sterile water. Sodium citrate was eliminated from the medium, (see page 274 for reasons), and consequently, it was designated DM$^-$.

Composition of D/S DM$^-$

K_2HPO_4	14.0g
KH_2PO_4	6.0g
$MgSO_4.7H_2O$	0.2g
$(NH_4)_2SO_4$	2.0g
Water	to 1 litre

Each compound was dissolved in water in the order shown, care being taken to ensure that each had thoroughly dissolved before the next was added. The volume was then made up to one litre, the solution decanted as 50ml volumes into screw-capped 'Medical Flats', and the bottles autoclaved at 121°C for 15 minutes. When cool, the bottles were stored at room temperature. The final pH of the medium was 7.0-7.1.

Buffers. Sörensens Phosphate Buffer (185), pH range 5.0-8.2, was used throughout this work as the standard diluent for the preparation of thiomersal test solutions. It was normally formulated as an isotonic solution of pH 7; isotonic buffers were also prepared at pH 5, 6 and 8. A further isotonic buffered solution at pH 4.5 was made by omitting the di-sodium hydrogen orthophosphate dihydrate. Tonicity investigations required the use of two further buffered solutions, one without any sodium chloride (hypotonic) and one with 0.9% w/v sodium chloride (hypertonic). For photochemical studies, EDTA was also included in the isotonic buffer at pH 7, at a concentration of 0.1% and 0.01% w/v. Table 1 provides the full range of buffered solutions used.

Test Solutions. Thiomersal was used at a concentration of 0.008% w/v as the standard test concentration. It was also used at 0.001%, 0.004% and 0.01% w/v in studies on the effect of concentration. All solutions were freshly prepared and immediately protected from light with aluminium foil. The solutions were stored at 4°C in the dark when not in use and discarded at the end of each day. If required sterile, the solution was filtered through a 0.22 μm membrane filter. It had been previously been shown in the Department (101), that no loss of thiomersal occurred with this method.

Other Materials

Membrane Filters. All membrane filtration was carried out through

TABLE 1

Formulation of Sörensen's Phosphate Buffers Used

Type	Compound	Concentration in g litre^{-1}	Concentration in Moles litre^{-1}
Hypotonic	KH_2PO_4	3.5594	2.61×10^{-2}
pH 7	$Na_2HPO_4.2H_2O$	7.2230	4.06×10^{-2}
Isotonic	KH_2PO_4	3.5594	2.61×10^{-2}
pH 7	$Na_2HPO_4.2H_2O$	7.2230	4.06×10^{-2}
	NaCl	4.4358	7.60×10^{-2}
Hypertonic	KH_2PO_4	3.5594	2.61×10^{-2}
pH 7	$Na_2HPO_4.2H_2O$	7.2230	4.06×10^{-2}
	NaCl	9.0000	1.54×10^{-1}
Isotonic	KH_2PO_4	9.0800	6.67×10^{-2}
pH 4.5	NaCl	5.0956	8.73×10^{-2}
Isotonic	KH_2PO_4	8.9710	6.59×10^{-2}
pH 5	$Na_2HPO_4.2H_2O$	0.1426	8.01×10^{-4}
	NaCl	5.0826	8.70×10^{-2}
Isotonic	KH_2PO_4	7.9632	5.85×10^{-2}
pH 6	$Na_2HPO_4.2H_2O$	1.4612	8.21×10^{-3}
	NaCl	4.9625	8.50×10^{-2}
Isotonic	KH_2PO_4	0.4994	3.67×10^{-3}
pH 8	$Na_2HPO_4.2H_2O$	11.2266	6.31×10^{-2}
	NaCl	4.0701	6.97×10^{-2}
Isotonic	KH_2PO_4	3.5594	2.61×10^{-2}
pH 7,	$Na_2HPO_4.2H_2O$	7.2230	4.06×10^{-2}
with 0.01%	NaCl	4.4118	7.55×10^{-2}
EDTA	EDTA	0.1000	2.69×10^{-4}
Isotonic	KH_2PO_4	3.5594	2.61×10^{-2}
pH 7,	$Na_2HPO_4.2H_2O$	7.2230	4.06×10^{-2}
with 0.1%	NaCl	4.1958	7.18×10^{-2}
EDTA	EDTA	1.0000	2.69×10^{-3}

Sartorius cellulose nitrate filters, type SM 113 (Sartorius - Membranfilter GmbH, West Germany). The following grades of filters were used:-

1) 25mm diameter, 0.22 μm pore size,

2) 47mm diameter, 0.22 μm pore size.

Petri Dishes. Pre-sterilised, 10cm diameter, polystyrene Petri dishes (Sterilin Products Ltd.) were used.

Polystyrene Universals. Pre-sterilised Universals (Sterilin Products Ltd.), were used where sterile containers of up to 30ml capacity were required.

Tips. Polypropylene tips (Oxford Laboratories Ltd.) were obtained in two sizes, with capacities of 1ml and 5ml. They were cleaned by boiling in distilled water and rinsing in three changes of cold water.

Sterilisation. The tips and other dry-heat sensitive items were packed in DHSS specification bags (DRG Hospital Supplies), sealed with a Rotacrimp Mk 2 (E.D.C. Electricals Ltd., Somerset), and placed in a Drayton Castle autoclave, (Sybron Corporation, Cambridge), for 4 minutes at 135^{o}C. Single membrane filters were sterilised by being packed between two sheets of filter paper in a DHSS specification bag, and autoclaved in a bench-top autoclave (Taylor Rustless Instruments Co. Ltd.) at 121^{o}C for 15 minutes. The Swinnex units were sterilised with membranes 'in-situ', in DHSS sterilisation bags in the bench-top autoclave.

Basic Instrumentation

pH Meter. All measurements of pH were made with a combined electrode model, PW9418 (Phillips). Standardisation of the meter was carried out prior to use with standard reference buffers at pH 4.0, 7.0 and 9.2. These were freshly prepared from buffer tablets (Fisons Ltd), and stored at 4^{o}C for up to one week.

Spectrophotometer. Optical density measurements of bacterial suspensions were made using a matched pair of quartz cuvettes in a SP600 spectrophotometer (Unicam Instruments, Cambridge). The 'blank' cuvette contained the suspending medium as the reference. Suspensions with an optical density (O.D.) of more than 0.2 O.D. units were diluted before being read.

Replicating Pipettes. The Oxford Triple Range Sampler with settings at 0.2ml, 0.5ml and 1.0ml, and the Macro Set Sampler with the range of 1.0ml to 5.0ml (Oxford Laboratories Ltd.), were used for measuring and diluting solutions in microbiological work. To determine the errors involved in their use, ten weighings of water at 20°C were recorded on an analytical balance at the required volume settings. A fresh tip was fitted on each occasion, and the Macro Set Sampler was also reset for each reading. Tables 2 and 3 show the mean delivery weights, the standard deviations and coefficients of variation for both Samplers.

Graduated Pipettes. A Pi pump GR10 (Glasfirn, Richardsons of Leicester Ltd.), fitted to a 10ml cylindrical, graduated, grade B pipette, was used to deliver 9.8ml of thiomersal test solution for bacterial challenge experiments. Table 4 shows the statistical analysis of the weights of ten such volumes of 9.8ml of water at 20°C, delivered from ten separate pipettes.

Thermometers. Temperature measurements were made with a total immersion thermometer, 'E-Mil' grade (Fisons Ltd.), with a range of -5° to +50°C, and subdivisions of 0.1°C.

Large Volume Filtration/Cell Harvesting Apparatus. A negative pressure, all glass, Pyrex, 47mm Filter apparatus (Millipore S.A., France), with the appropriate membrane fitted, was used for the harvesting of bacterial cells. This unit has a fritted glass, membrane support. When ultra-clean filtrates were essential, the model with a removable stainless

TABLE 2

Weights (grams) of water delivered from the Oxford Triple Range Sampler with a fixed volume setting

Replicate Number	Volume Setting 0.2ml	0.5ml	1.0ml
1	0.2035	0.5031	0.9913
2	0.2021	0.5060	1.0064
3	0.2040	0.5069	1.0030
4	0.2037	0.5044	1.0039
5	0.2014	0.5039	1.0021
6	0.2012	0.5043	1.0017
7	0.2020	0.5032	1.0004
8	0.2061	0.5001	0.9997
9	0.2040	0.5020	1.0027
10	0.2036	0.5031	1.0036
Mean Weight	0.2032	0.5037	1.0015
Standard Deviation	0.0015	0.0019	0.0040
Coefficient of Variation	0.73%	0.38%	0.40%
Theoretical Weight of Water at 19.8°C	0.1997	0.4991	0.9982
% Difference obtained from Theoretical Weight	+1.75%	+0.92%	+0.33%

Conclusion

The Sampler was suitable for its intended use.

TABLE 3

Weights (grams) of water delivered from the Oxford Macro Set Sampler with variable volume setting

Replicate Number	Volume Setting 3.8ml	4.0ml	4.5ml
1	3.7888	3.9902	4.5023
2	3.7937	3.9877	4.4949
3	3.8045	4.0192	4.5116
4	3.7782	4.0101	4.5146
5	3.8041	4.0026	4.4937
6	3.7854	4.0208	4.4955
7	3.7949	4.0117	4.5037
8	3.8156	4.0110	4.4906
9	3.8077	3.9890	4.5019
10	3.8098	4.0127	4.5080
Mean Weight	3.7938	4.0055	4.5017
Standard Deviation	0.0119	0.0124	0.0080
Coefficient of Variation	0.31%	0.31%	0.18%
Theoretical Weight of Water at 19.8°C	3.7933	3.9929	4.4921
% Difference obtained from Theoretical Weight	+0.13%	+0.31%	+0.21%

Conclusion

The Sampler was suitable for use at these volume settings.

TABLE 4

Weights (grams) of 9.8ml water delivered from 10.0ml graduated, grade B pipettes, using a Pi Pump

Replicate Number	Volume Setting: 9.8ml
1	9.8093
2	9.8026
3	9.8043
4	9.8125
5	9.8198
6	9.8204
7	9.8193
8	9.8019
9	9.8285
10	9.9132
Mean Weight	9.8132
Standard Deviation	0.0088
Coefficient of Variation	0.09%
Theoretical Weight of Water at 19.8°C	9.7824
% Difference from Theoretical Weight	+0.32%

Conclusion

These pipettes were suitable for delivering 9.8ml when required.

steel support was used.

Small Volume Filtration Apparatus. Sterile Swinnex Filter holders (Millipore S.A., France) fitted with membranes were used for cleaning and sterilising small volumes of liquid dispensed with a hypodermic syringe.

Shaking Water Baths. A Grant shaking water immersion bath, type SS30 (Grant Instruments, Cambridge Ltd.), was used for the incubation of microbiological liquid cultures. This was set at 37°C, with a shaking speed of 120 strokes $minute^{-1}$. The temperature fluctuations of the bath were monitored by taking daily measurements over a week, at the same time each day. These readings were found to range between 36.8-37.0°C. The water bath was protected by a built-in safety cut-out system, set at 50°C.

Static Water Baths. All controlled temperature experiments were carried out in a 20 litre stainless steel, insulated tank, heated with a Grant Immersion heater, type SU6 (Grant Instruments, Cambridge Ltd.). This unit also incorporated a built-in safety cut-out system against over-current, overtemperature and low liquid level. A cooling unit, 'U-Cool' (Utile Products, Nestlab Instruments Inc.), was used in conjunction with the heater for temperatures of 20°C and below. Monitoring temperature readings here revealed no fluctuations when the 'U-Cool' was in use. When the heater was set at 25°C, the temperature readings recorded ranged between 24.8-25.1°C.

Microbiological Methods

Test Organisms

Organisms 1-4 were obtained from the National Collection of Type cultures (NCTC). Organism 5 was kindly provided by the London School of Hygiene and Tropical Medicine (LSHTM), and organism 6 was available in the Department.

1) Staph. aureus NCTC 6571 (ATCC 9144, NCIB 6571, NRRL-B314).
   This strain, sometimes referred to as the 'Oxford strain' is recommended in the catalogue of the NCTC as being suitable for antibiotic assays and sensitivity testing.
2) Ps. aeruginosa NCTC 6750 (ATCC 19429, NCIB 6750).
   This strain is the suggested working type. It was supplied to the NCTC in 1944 by the Lister Institute.
3) Ps. aeruginosa var. erythrogenes NCTC 6749.
   Originally isolated in 1944, this is the recommended disinfectant testing strain. It was included as an additional test organism.
4) E. coli NCTC 86 (ATCC 4157, NCIB 86)
   This was originally isolated in about 1885, but was not deposited at the NCTC until 1920. It is the recommended strain for disinfectant testing.
5) C. albicans LSHTM 3153.
6) B. subtilis FD TEMP
   This is the Fort Detrick strain, Maryland, USA. It was originally received from D.W. Tempest, Porton, UK, who reported it as probably being the same as ATCC 9372. It was subsequently characterised by R.J. Hobbs (186), and is the organism generally used in its spore form as a marker for dry-heat sterilisation.

Characteristics of Test Organisms

Organisms 1-5 were obtained as freeze-dried specimens. Tryptone Soy Broth (TSB) was used as the suspending medium, and the organisms were streaked out on Tryptone Soy Agar (TSA) plates. Highly concentrated, washed spore suspensions of B. subtilis FD TEMP were available, and these were used to inoculate a tube of TSB and to streak out a TSA plate. All tubes and plates were incubated at 37°C for 24 hours. Gram stains (184) were then made from the broth cultures, to verify the Gram reaction and to check if any contamination had occurred. Microscopic examinations of wet films were also made to determine the salient features of the living organisms. For C. albicans, the film was stained with Löffler's methylene blue prior to examination. Motility was detected by the 'hanging-drop' method (184). The TSA plates were then used to record typical colonial morphology. (The C. albicans plate was incubated for 48 hours at 37°C, as after 24 hours, growth was too limited for details of the colonies to be easily seen). Table 5 summarises the results of these observations.

Maintenance of Test Organisms

Master cultures of organisms 1-5 in TSB were stored in liquid nitrogen at -196°C, and working cultures made by streaking out pairs of TSA slopes. After incubation at 37°C for 48 hours, these were kept at 4°C. The stock slopes were subcultured monthly for three months, after which a fresh master culture was put into use. The spore suspension of B. subtilis FD TEMP was kept at 4°C, and TSA slopes were inoculated from this as above.

Commercially Available Growth Media

The following commercially available media were used:-

TABLE 5

Characteristics of the Test Organisms.

Organism	Microscopic Examination	Colonial Morphology (24h at 37°C on TSA)*
Staph. aureus NCTC 6571	Gram-positive eocci, arranged in clusters; non-motile.	Opaque, low convex, glossy, circular, entire-edged colonies. Size: 2-4mm; Colonies: golden yellow; Medium: unpigmented. Slight odour.
Ps. aeruginosa NCTC 6750	Gram-negative slender rods; non-motile.	Low convex, irregular, translucent, spreading edged, with dark green centre; lysogenic. Size: 4-6mm; Medium: green/ light brown round colonies. Strong characteristic odour.
Ps. aeruginosa var. erythrogenes NCTC 6749	As above, but motile.	As above, but colonies are brown. Medium: dark brown. Strong amine smell.
E. coli NCTC 86	Gram-negative rods; motile.	Translucent, circular, entire-edged, flat colonies. Size: 2mm; Colonies: creamy beige; Medium: unpigmented. Strong odour.
C. albicans LSHTM 3153	Gram-positive, ovoid cells in clumps; some pseudo-mycelium; no chlamydospores observed.	Opaque, umbonate, crenated-edged colonies. Size: 2-3mm; Colonies: cream with deep cream centres; Medium: unpigmented. Strong characteristic odour.
B. subtilis FD TEMP	Gram-positive round-ended rods, central spores; non-motile.	Opaque, umbonate, crenated-edged colonies. Size: 4-6mm; Colonies: pale orange, deep orange centres. Medium: unpigmented. Mild odour.

* 48h at 37°C for C. albicans.

Type	Code	Supplier	Wt(g) litre^{-1}	Final pH
Tryptone Soy Agar (TSA)	lab 11	London Analytical and Bacteriological Media Ltd. (Lab M Ltd.)	37	7.3
Tryptone Soy Broth (TSB)	lab 4	Lab M Ltd.	30	7.3
Nutrient Agar (NA)	CM 3	Oxoid Ltd.	28	7.4
Columbia Agar Base (CAB)	lab 1	Lab M Ltd.	44	7.3
Malt Extract Agar (MEA)	CM 59	Oxoid Ltd.	50	5.4
Agar No. 3	L 13	Oxoid Ltd.	12	-

Preparation of Stock Slopes

These were prepared from TSA by weighing out 37 grams and adding the powder to a litre of water. After 15 minutes to allow the agar to rehydrate, the mixture was boiled to dissolve the agar. About 12ml volumes of agar were then poured into McCartney bottles fitted with rubber-lined screw caps, and autoclaved at 121^{o}C for 15 minutes. When the temperature in the autoclave had dropped to 60^{o}C, the McCartneys were removed and allowed to cool at an incline, to form the required slope. Care was taken to ensure that no agar set too far into the neck of each bottle.

Preparation of Plating Media

The specified quantity of the appropriate agar was added to a litre of water and allowed to stand for 15 minutes. After being dissolved in

freely-flowing steam, the agar was autoclaved under normal conditions, apart from with MEA, where the manufacturer's conditions were 115°C, for 10 minutes. When the temperature in the autoclave had cooled to about 60°C, nominal 20ml volumes were poured into Petri dishes. The agar was allowed to set and the plates were then stored in the dark at 4°C. No plates were kept longer than 4 days. Prior to use, the plates were overdried under ventilated conditions at 37°C for 75 minutes. Agar No. 3 was used where a setting agent for the chemically defined media was required. The latter was prepared as a double-strength, sterile solution, (see page 64), and the agar suspension was sterilised by autoclaving. When the temperature in the autoclave had dropped to about 60°C, the gel was removed and added aseptically to the defined media. After thorough mixing, this was used to prepare the plates as outlined above.

Preparation of TSB

TSB was used as a general purpose, liquid, culture medium, where needed. It was prepared by dissolving 30g of granules in a litre of warm water. 100ml volumes were then poured into 'Medical Flats'; screw caps were fitted and the broth autoclaved at 121°C for 15 minutes. When cool, the bottles were removed from the autoclave and stored at room temperature.

Chemically Defined Growth Media

A synthetic growth medium (SGM) based on that of Mah, Fung and Morse (187), had previously been developed in the Department for the growth of Staph. aureus NCTC 6571 (188). Preliminary growth experiments carried out with SGM revealed that it had several disadvantages:-

1) when the primary culture was inoculated from a slope, growth

normally only occurred at about 72 hours. Growth did not always take place at 48 hours, and if it did occur, was extremely poor, despite the presence of 12 amino acids,

2) the media was faintly cloudy on preparation, and this precipitation increased with time.

It was decided, therefore, to find a medium that would reduce the primary cycle to 24 hours, as well as give luxuriant growth, and to eliminate the precipitation problem if possible. It also seemed desirable to base this new medium on one of the more common minimal salts available, so that washing and other diluting operations could be carried out in the basal medium, minus the carbon source. The least possible trauma normally associated with these manipulations would then be exerted on the micro-organism. Two possible minimal salts media were:-

1) that of Davis and Mingioli (184), designated DM,

2) that of Anderson (189), designated M9.

Details of the growth experiments and the rationale for which minimal salts medium was finally selected, are outlined in Appendix I (page 274). The compositions of the complete basal medium and of the vitamin and amino acid supplement are shown in Tables 6, 7 and 8 respectively. Their methods of preparation are described below.

Preparation of Defined Media

This was prepared from its constituents in several stages.

1) Preparation of Basal Medium (BM)

500ml of a stock trace element solution (184) was prepared from the following compounds:-

TABLE 6

Composition of Basal Medium

Constituents	Amount litre^{-1}	Moles litre^{-1}
Carbon Source		
Glucose	10.0g	5.55×10^{-2}
Inorganic Salts		
K_2HPO_4, anhydrous	7.0g	4.02×10^{-2}
KH_2PO_4	3.0g	2.20×10^{-2}
$MgSO_4.7H_2O$	0.1g	4.06×10^{-4}
$(NH_4)_2SO_4$	1.0g	7.57×10^{-3}
Trace Elements		
$FeSO_4.7H_2O$	0.0005g	1.80×10^{-6}
$ZnSO_4.7H_2O$	0.0005g	1.70×10^{-6}
$MnSO_4.4H_2O$	0.0005g	2.20×10^{-6}
H_2SO_4, 0.1N	0.01ml	—

TABLE 7

Composition of Vitamin Solution

Compound	g litre^{-1}	Moles litre^{-1}
Thiamine Hydrochloride, anhydrous	0.00012	3.56×10^{-7}
Nicotinic Acid	0.0012	9.75×10^{-6}
Biotin	0.000008	3.27×10^{-8}
Folic Acid	0.000048	1.09×10^{-7}
Pyridoxal Hydrochloride	0.00008	3.93×10^{-7}

TABLE 8

Composition of Amino Acid Solution

Compound	g litre^{-1}	Moles litre^{-1}
L-Arginine monohydrochloride	0.025	1.19×10^{-4}
L-Aspartic acid	0.025	1.88×10^{-4}
L-Cysteine hydrochloride	0.025	1.59×10^{-4}
Glycine	0.025	3.33×10^{-4}
L-Histidine monohydrochloride	0.025	1.19×10^{-4}
L-Leucine	0.025	1.91×10^{-4}
L-Methionine	0.025	1.67×10^{-4}
L-Phenylalanine	0.025	1.51×10^{-4}
L-Proline	0.025	2.17×10^{-4}
L-Valine	0.025	2.13×10^{-4}

Component	Amount
$FeSO_4.7H_2O$	0.25g
$ZnSO_4.7H_2O$	0.25g
$MnSO_4.4H_2O$	0.25g
H_2SO_4, 0.1N	5ml
Water	to 500ml

These were dissolved sequentially in 450ml of water, the sulphuric acid added and the volume made up to 500ml. The stock solution was kept in the dark at 4^oC.

The following compounds were dissolved in 450ml of water in the order shown, some pre-warmed water being used to dissolve the glucose.

Component	Amount
K_2HPO_4	7.0g
KH_2PO_4	3.0g
$MgSO_4.7H_2O$	0.1g
$(NH_4)_2SO_4$	1.0g
Glucose	10.0g
Trace element solution	5ml
Water	to 500ml

After the trace element solution had been added, the pH of the solution was adjusted to 7.0-7.2 pH units if necessary, and the volume then made up to 500ml.

2) Preparation of Vitamin Supplement (S1)

This was prepared in two stages as shown below:-

a) Biotin - Folic Acid Solution

Component	Amount
Biotin	4mg
Folic Acid	24mg
Water	to 10ml

The vitamins were dissolved in about 6ml of water made alkaline with a few drops of sodium hydroxide. The pH was then adjusted to 7.0-7.2 pH units, and the volume made up to 10ml.

b) The complete vitamin solution was then prepared from the following components:-

Component	Amount
Thiamine Hydrochloride	6mg
Nicotinic Acid	60mg
Pyridoxal Hydrochloride	4mg
Biotin - Folic Acid Solution	1ml
Water	to 100ml

The remaining compounds were dissolved in 90ml water, the biotin - folic acid solution added, and the pH adjusted to 7.0-7.2 pH units. The volume was then made up to 100ml, and the solution sterilised by filtration through a 0.22 μm membrane in a Swinnex unit, using a glass syringe and a stainless steel needle to deliver 1ml of the sterile solution directly into sterile ampoules. The remaining biotin - folic acid solution was also sterilised by filtration; 1ml volumes were delivered into ampoules and kept as stock. All filtration procedures were carried out in a laminar flow cabinet. The ampoules were sealed in a flame, wrapped in foil, and stored in a deep freeze at -20^{o}C. Two ampoules of vitamin solution were used per litre of medium, when indicated.

3) Preparation of Amino Acid Supplement (S2)

The following amino acids were used:-

L-Arginine monohydrochloride (Arg)

L-Aspartic acid (Asp)

L-Cysteine hydrochloride (Cys)

L-Glycine (Gly)

L-Histidine monohydrochloride (His)

L-Leucine (Leu)

L-Methionine (Met)

L-β-Phenylalanine (Phe)

L-Proline (Pro)

L-Valine (Val)

25mg of each amino acid were dissolved in 90ml of pre-warmed water made alkaline with a few drops of sodium hydroxide. The solution was adjusted to a pH of 7.0-7.2, and made up to 100ml.

4) Preparation of Casamino Acid (vitamin-free) Supplement (S3)

Where specified, this was prepared by dissolving 10g of the product in 100ml of pre-warmed water.

5) Preparation of Complete Medium

Sterilisation of the complete medium was carried out by filtration through a 0.22 μm membrane, using the stainless steel support in the 47mm filtration unit. This was done in the laminar flow cabinet. Preliminary growth studies showed that the final composition of the media had to be varied with the micro-organism concerned, as shown in Table 9. The preparation technique was always the same in that the solutions were filtered in the order shown, namely, BM first, followed by S2 or S3. Using aseptic technique, the sterilised medium was transferred to a sterile litre flask, S1 added if required, and the volume made up to a litre with sterile water. The final medium was dispensed aseptically as 50ml volumes into sterile 'Medical Flats', and stored in the dark at 4^{o}C for up to three weeks.

TABLE 9

Organism - Defined Medium Formulation

Organism	Defined Medium
Ps. aeruginosa sp.	BM
E. coli NCTC 86	BM
Staph. aureus NCTC 6571	BM + S1 + S2
B. subtilis FD TEMP	BM + S1 + S2
C. albicans LSHTM 3153	BM + S1 + S3

BM Basal Medium

S1 Vitamin Supplement

S2 Amino Acid Supplement

S3 Casamino Acid (Vitamin-free) Supplement

Recovery Medium

A Recovery Medium (RM) was used to inactivate the thiomersal carried over in challenge testing experiments, and thus allow the growth of any surviving organisms. It was based on that of Norton et al (53), but the thioglycollate concentration was increased to 1.0% w/v when it was found that the original concentration of 0.1% w/v was insufficient to inactivate the higher concentrations of thiomersal used. (See Appendix II, page 279). The medium was normally prepared in two litre batches.

Constituents	% w/v
Sodium thioglycollate	1.0
TSB	3.0
'Tween' 80	3.0
Lecithin, egg about 90%	0.2
Water	to 100

Preparation of Recovery Medium

The sodium thioglycollate and TSB granules were dissolved sequentially in about 300ml of warm water. The lecithin was placed in a glass mortar, and allowed to come to room temperature before a few drops of hot water were added. After a few minutes to allow the lecithin to rehydrate, the 'Tween' 80 was added, a few ml at a time, and mixed in with a glass pestle. When all the 'Tween' 80 had been used and the mixture was homogenous, it was transferred to 500ml of hot water in a beaker, and agitated thoroughly before being added to the sodium thioglycollate - TSB solution. The volume was then made up to two litres with water, and the complete medium dispensed as 100ml volumes into 'Medical Flats' with screw caps. Autoclaving was carried out under normal conditions. When the temperature in the autoclave had dropped to about 70°C, the bottles were removed and inverted several times to resuspend the separated brown layer at the base of each bottle. This shaking process was repeated until the medium was clear. The bottles were stored at room temperature in the dark, and were used within 3 weeks of preparation.

Tests on Efficacy of Recovery Medium

In order to verify that the Recovery Medium was reliable, several tests were carried out to ensure that micro-organisms were recovered when present in low numbers, despite high concentrations of thiomersal, and also that the RM itself exerted no deletrious effect on the cells. These tests are outlined below.

1) 'Direct' Test - Tube Method. This was carried out as described by Norton et al (53), using the highest working concentration of thiomersal, 0.01% w/v. 0.5ml of a suspension of the appropriate organism in DM$^-$ prepared to contain about 20 colony forming units ml^{-1} (cfu ml^{-1}),

(see page 77 for technique), was added to each of two tubes containing 9ml of RM and 0.5ml of 0.01% w/v thiomersal. After mixing, the tubes were incubated at 37°C for 48 hours. This was repeated for all the test organisms.

In all cases, growth was obtained within 48 hours, though with C. albicans, incubation with shaking was necessary for observable turbidity at this time. All tubes were streaked out to verify that growth was not due to contaminating organisms. The medium was thus able to allow the growth of an inoculum of as low as 10 organisms, after the thiomersal had been inactivated.

2) 'Indirect Test - Plate Method. A stock suspension of the organism in DM^- was prepared, and further dilutions made to give three suspensions containing about 1×10^6, 1×10^4 and 1×10^3 cfu ml^{-1}. At time '0', 0.2ml of a 0.01% w/v solution of thiomersal was added to 3.6ml of RM in a tube. This was agitated and immediately 0.2ml of the suspension containing 1×10^6 cfu ml^{-1} was added. After further agitation, the, tube was set aside for 10 minutes, before being serially diluted in DM^- and plated out. This was repeated for the other two suspensions, plates being spread from the RM tube for the 1×10^4 cfu ml^{-1} challenge level, and 0.5ml being spread from the RM tube for the 1×10^3 cfu ml^{-1} initial challenge level. Positive controls were set up by repeating the procedure, but replacing the thiomersal with DM^-. The entire procedure was repeated for all the main test organisms. The results for two representative Gram-positive and Gram-negative bacteria are shown in Tables 10 and 11.

Effect of Length of Time Incubated in RM before Diluting/Plating Operations.

The preceeding tests confirm the suitability of the RM to be used in

TABLE 10

Plate tests for determining the efficiency of the Recovery Medium.

Test organism: Staph. aureus NCTC 6571.

Incubation conditions: 24 hours at 37°C.

	Experimental		Control		Student 't' test (n=10)	
Dilution Factor	Mean viable count	cfu ml^{-1}	Mean viable count	cfu ml^{-1}	Tab	Cal
10^4	101.4	1.01×10^6	105.4	1.05×10^6	2.75	1.27
10^2	98.6	9.86×10^3	103.2	1.03×10^4	2.75	1.56
4×10^1	24.4	9.76×10^2	25.4	1.02×10^3	2.75	0.39

TABLE 11

Plate tests for determining the efficiency of the Recovery Medium.

Test organism: Ps. aeruginosa NCTC 6750.

Incubation conditions: 24 hours at 37°C.

	Experimental		Control		Student 't' test (n=10)	
Dilution Factor	Mean viable count	cfu ml^{-1}	Mean viable count	cfu ml^{-1}	Tab	Cal
10^4	104.0	1.04×10^6	105.2	1.05×10^6	2.75	0.51
10^2	106.6	1.07×10^4	103.6	1.04×10^4	2.75	1.27
4×10^1	25.2	1.01×10^3	23.0	9.20×10^2	2.75	1.04

Conclusions

The means in both Tables were not significantly different at the 5% level, so that the RM was shown to be effective.

inactivating thiomersal. However, the organisms had only been in momentary contact with thiomersal, and it is possible that with longer exposures, 10 minutes in the RM might not be sufficient.

To investigate this, a challenge experiment was set up, using thiomersal at a concentration of 0.01% w/v. The test solution was inoculated with the appropriate bacterial suspension, and the challenge mixture placed in the 25°C water bath for exposures that varied with the test bacteria. At the desired time, 0.2ml of the challenge mixture was removed to a tube containing 3.8ml RM. After mixing, this was immediately diluted and plated out. At 5, 10, 15, 20 and 30 minutes, the diluting and plating operations from the RM tube were repeated. All plates were incubated as normal at 37°C.

The results for Staph. aureus NCTC 6571 and Ps. aeruginosa NCTC 6750, together with a statistical analysis, are shown in Tables 12 and 13. It can be seen that an increased holding time in the RM does not lead to an increase in the numbers of survivors recovered for both organisms. As a result, a standard contact time of 10 minutes was adopted for all subsequent challenge experiments.

Inoculation of Cultures

The stock slopes of the organism was used to streak a TSA plate (BM solidified with Agar No. 3 for Ps. aeruginosa NCTC 6750). This was then incubated at 37°C for 48 hours. From this, a primary (1^{o}) culture was initiated by touching the surface of an isolated, typical colony with a loop, and using this to inoculate 50ml of the appropriate, pre-warmed defined medium contained in an Erlenmeyer flask. The flask was placed in the water bath and incubated with shaking for exactly 24 hours. A secondary (2^{o}) liquid subculture was then made by pipetting 1ml of the 1^{o} culture into a fresh flask of pre-warmed defined medium. This 2^{o}

TABLE 12a

Effect of varying length of time incubated in RM before plating.

Test Organism: Staph. aureus NCTC 6571, exposed to thiomersal for 6 hours.

Concentration of thiomersal: 0.01% w/v.

Minutes in RM	Viable Count					Mean	cfu ml^{-1}
<1	153	152	147	157	155	152.8	1.53×10^6
5	143	157	152	151	142	149.0	1.49×10^6
10	144	141	142	148	162	147.4	1.47×10^6
15	159	140	154	153	153	151.8	1.52×10^6
20	138	142	147	153	155	147.0	1.47×10^6
30	159	150	150	139	143	148.2	1.48×10^6

Dilution factor: 10^4

TABLE 12b

Analysis of Variance

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Squares	Variance Ratio
Treatments	5	143.37	28.67	0.59
Residual	24	1153.60	48.07	
Total	29			

$F_{0.05}$ (5,24) = 2.62 $F_{0.01}$ (5,24) = 3.90

Conclusion

The result is not significant at the 5% or 1% levels

TABLE 13a

Effect of varying length of time incubated in RM before plating.

Test organism: Ps. aeruginosa NCTC 6750, exposed to thiomersal for 1 hour.

Concentration of thiomersal: 0.01%

Minutes in RM	Viable Count					Mean	cfu ml^{-1}
<1	82	90	92	90	81	87.0	8.70×10^5
5	92	94	88	91	88	90.6	9.06×10^5
10	91	93	94	97	96	94.2	9.42×10^5
15	88	91	88	96	93	91.2	9.12×10^5
20	99	90	83	91	89	90.4	9.04×10^5
30	92	94	94	96	86	92.4	9.24×10^5

Dilution factor: 10^4

TABLE 13b

Analysis of Variance

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Squares	Variance Ratio
Treatments	5	143.77	28.75	1.76
Residual	24	391.20	16.30	
Total	29			

$F_{0.005}(5,24) = 2.62$ $F_{0.01}(5,24) = 3.90$

Conclusion

The result is not significant at the 5% or 1% levels.

culture was then used for the harvesting of cells in late stationary phase, at exactly 22 hours. Cells of this age were used for all challenge experiments unless otherwise indicated.

Harvesting of Test Micro-organisms

Cells were harvested using the all glass filter apparatus. A sterile 47mm membrane filter of pore size 0.22 μm was placed on the sintered glass support with alcohol-flamed forceps. Negative pressure was then applied and the filter rinsed with four 5ml volumes of DM^-. The required volume of 2^o culture, normally 1ml, (10ml for C. albicans), was pipetted onto the centre of the filter and a second washing sequence carried out. The vacuum was then released and the filter transferred to a boiling tube containing 10ml of DM^- using aseptic technique. The tube was thoroughly agitated with a Whirlimixer (Fisons Ltd.), to resuspend the cells.

Calibration Curves and Viable Count Determinations

The stock suspension of the orgainsm in DM^- was prepared as described, sufficient culture being filtered to give an initial O.D. reading of about 0.2 O.D. units. This would enable a range of dilutions to be made, to obtain readings of between 0.05 and 0.2 O.D. units. The latter was selected as the upper limit for readings, because it had previously been found (Anthony - unpublished data) that above this O.D., plots ceased to be linear for Staph. aureus and Ps. aeruginosa. It was decided, therefore, to restrict readings for the other test organisms to this range. The O.D. of 3ml of each appropriate dilution was then read on the SP 600, with DM^- as the 'blank' in the reference cuvette. The wavelength used was 470nm for all organisms apart from Ps. aeruginosa, which was read at 600nm. A viable count determination was then under-

taken for each dilution, to relate each reading to its corresponding count. For this purpose, ten-fold serial dilutions in DM^{-} were made, whirlimixing at each dilution stage, until a dilution was reached which would give about 500 cfu ml^{-1}. After preliminary trials, it was established that the following dilutions were suitable for the range of O.D. readings used:-

Organism	Dilution
Staph. aureus NCTC 6571	-5
Ps. aeruginosa NCTC 6750	-5/-6
E. coli NCTC 86	-5
C. albicans LSHTM 3153	-3/-4
B. subtilis FD TEMP	-5

The surface spread method of determining the viable count was used and this involved spreading 0.2ml volumes of the final dilution evenly over the surface of each of five pre-dried TSA plates, with a sterile glass spreader. Any surplus fluid was allowed to soak in before the plates were inverted and incubated at 37°C for 24 hours; (48 hours for C.albicans). The number of colonies on each plate was then counted with a colony counter (A. Gallenkamp & Co Ltd.), and the mean value calculated. The number of viable bacteria in the original suspension was then obtained after multiplying the mean count by the dilution factor. A graph of O.D. units against cfu ml^{-1} was then plotted. This procedure was repeated for each of the main test organisms. Figures 1 to 5 (Appendix III, pages 283 to 285) illustrate the relationships between O.D. and cfu ml^{-1}.

Estimation of Errors Involved in Viable Counting. Errors in viable counting mainly arise from two sources:-

1) through the use of the replicating pipettes,
2) through inefficient mixing of the suspension at each dilution stage and poor spreading techniques.

Statistical analyses of the former have shown that both Oxford Samplers are suitable for their designated use (Pages 55 and 56). To test the latter, five viable count determinations were carried out on an experimental suspension containing 5×10^7 cfu ml^{-1}, using a separate dilution for each. Two representative Gram-positive and Gram-negative bacteria were chosen from the main test series and the results obtained, together with an analysis of variance in each case, are shown in Tables 14 and 15.

Challenge Testing

9.8ml of thiomersal test solution at the desired concentration, were pipetted into a boiling tube using a graduated glass pipette fitted with a GR 10 Pi Pump (Glasfirn and Richardsons of Leicester Ltd.). The boiling tube was placed in the water bath set at the desired working temperature and allowed to equilibrate. Light was excluded from test solutions during an experiment by the use of aluminium foil. A control tube was set up by pipetting 9.8ml of the appropriate diluent into an identical tube and allowing that to equilibrate as well. A stock suspension of the test organism in DM^- was prepared as described, to contain 5×10^7 cfu ml^{-1}. This was also allowed to equilibrate in the water bath.

At time '0', the bacterial suspension was whirlimixed thoroughly, and 0.2ml withdrawn to the thiomersal tube. After further mixing, 0.2ml was pipetted into 3.8ml of RM, agitated and allowed to stand for 10 minutes before being serially diluted and plated out. This procedure was repeated with the control tube. At suitable time intervals thereafter, (times which varied with the organism concerned), appropriate volumes were withdrawn from the challenge mixture, inactivated in RM,

TABLE 14a

Errors associated with the homogenity of suspensions of Staph. aureus.

Dilution Series	Viable Count				
1	102	110	112	110	101
2	112	114	108	111	108
3	111	113	114	117	116
4	108	111	108	116	113
5	114	100	118	111	114

Dilution Factor: 10^4

TABLE 14b

Analysis of Variance.

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Squares	Variance Ratio
Treatments	4	132.64	33.16	1.71
Residual	20	388.00	19.40	
Total	24			

$F_{0.05}\ (4,20) = 2.87$ $F_{0.01}\ (4,20) = 4.43$

Conclusion

Result is not significant at the 5% or 1% levels.

TABLE 15a

Errors associated with the homogenity of suspensions of Ps. aeruginosa.

Dilution Series	Viable Count				
1	100	105	99	105	102
2	97	99	104	105	100
3	107	100	105	102	98
4	102	107	108	104	100
5	100	96	104	105	99

Dilution Factor: 10^4

TABLE 15b

Analysis of Variance.

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Squares	Variance Ratio
Treatments	4	37.04	9.26	0.81
Residual	20	229.60	11.48	
Total	24			

$F_{0.05}\ (4,20) = 2.87$ $F_{0.01}\ (4,20) = 4.43$

Conclusion

Result is not significant at the 5% or 1% levels.

diluted if necessary and plated out. The control tube was normally only plated out again at the end of the experiment, to determine the tolerance of the organism to the respective diluent and environmental conditions; if the experiment continued over several days, however, a daily sampling was normally carried out. All plates were incubated at 37°C, though the length of incubation was adjusted to meet the needs of the organism concerned.

Organism	Incubation Protocol
Ps. aeruginosa spp. *E. coli* NCTC 86 *B. subtilis* FD TEMP	Plates counted after 1 day; reincubated for a further day.
Staph. aureus NCTC 6571	Plates counted at 2 days; reincubated a further 2 days.
C. albicans LSHTM 3153	Plates counted after 4 days; reincubated a further 2 days.

Where possible, only plates with between 30 and 300 colonies were counted. Any changes in colonial morphology with respect to the control plates were also noted.

Where preliminary experiments had indicated that the numbers of survivors had fallen to 100 cfu ml^{-1} and below, the membrane filtration technique was employed to concentrate the numbers of survivors. This involved taking a sample as normal and placing it in the RM. However, instead of plating out from this tube, a 0.5ml sample was filtered through a sterile 0.45 μm pore size membrane filter, under negative pressure. The vacuum was carefully released and the filter transferred with alcohol-flamed forceps to a TSA plate that had been pre-dried for about 15 minutes, merely to remove surplus surface water. Sterilisation

of contaminated surfaces between samples was achieved by alcohol-flaming. (A sample of sterile water was filtered through to hasten cooling of the flamed surfaces, where necessary). All plates were incubated as normal, but left in the upright position. The advantage that this technique has is that the sample volume filtered can be increased as the number of survivors falls. When survivors are in very low numbers, the entire contents of the RM tube can be filtered, thus increasing the probability of finding the odd survivor. Tables 16 and 17 compare the results obtained by the surface spread method with the membrane filtration technique, when the numbers of cfu ml^{-1} are about 100.

TABLE 16

Comparison of ability of surface spread method and membrane filtration technique to recover low numbers of survivors.

Test Organism: Staph. aureus NCTC 6571.

Incubation Conditions: 48h at $37^{o}C$.

Surface Spread Method			Membrane Filtration			Student 't' test (n=10)	
Dilution Factor	Mean viable count	cfu ml^{-1}	Dilution Factor	Mean viable count	cfu ml^{-1}	Tab	Cal
5	17.6	8.80×10^{1}	2	41.0	8.20×10^{1}	2.75	1.52

Conclusion

The means were not significantly different at the 5% level, so that membrane filtration could be used for viable count determinations when low numbers of survivors were anticipated.

TABLE 17

Comparison of ability of surface spread method and membrane filtration technique to recover low numbers of survivors.

Test Organism: Ps. aeruginosa NCTC 6750.

Incubation Conditions: 48h at 37°C.

Surface Spread Method			Membrane Filtration			Student 't' test (n=10)	
Dilution Factor	Mean viable count	cfu ml^{-1}	Dilution Factor	Mean viable count	cfu ml^{-1}	Tab	Cal
5	18.0	9.00×10^{1}	2	43.4	8.68×10^{1}	2.75	1.29

Conclusion

The means were not significantly different at the 5% level, so that membrane filtration could be used for viable count determinations when low numbers of survivors were anticipated.

Photochemical Methods

The Light Source

This was supplied by eight Thorn 450mm, 15 watt, quick-start, Northlight, colour-matching fluorescent tubes (Wilts. Wholesale Electrical Co. Ltd.). The spectral characteristics of these have been shown (190). The tubes were positioned evenly on either side of the walls of a wooden box of dimensions: 530mm x 530mm x 590mm. The box had been painted white to increase the intensity of reflected light. A wooden block fixed to the base of the box, was fitted with two ampoule-holding racks. The block had been fixed so that the base of an ampoule was in line with the lowest fluorescent tube. Figure 6 is a diagrammatic representation of the light box and a typical irradiation experiment in progress can be seen in Plate 1. Temperature measurements were taken before the start of each irradiation experiment, and whenever ampoules were removed. The temperatures were found to range between 27.5-37.0^{o}C, with a mean temperature of 33.5^{o}C.

It has been shown that in the first 100 hours of its life, the output of a fluorescent tube falls by up to five per cent (190). Thereafter, the rate is slower until at 2000 hours, it has fallen by a further five to ten per cent, depending on the lamp rating and the phosphor mix. Calibrated thermopile readings made in the light box (101), showed that there was no significant difference in the light intensity values obtained over a period ranging from Week 3 to Week 11 in the life of a fluorescent tube. All irradiation studies were consequently carried out during this period. The tubes were left on continuously during the test series.

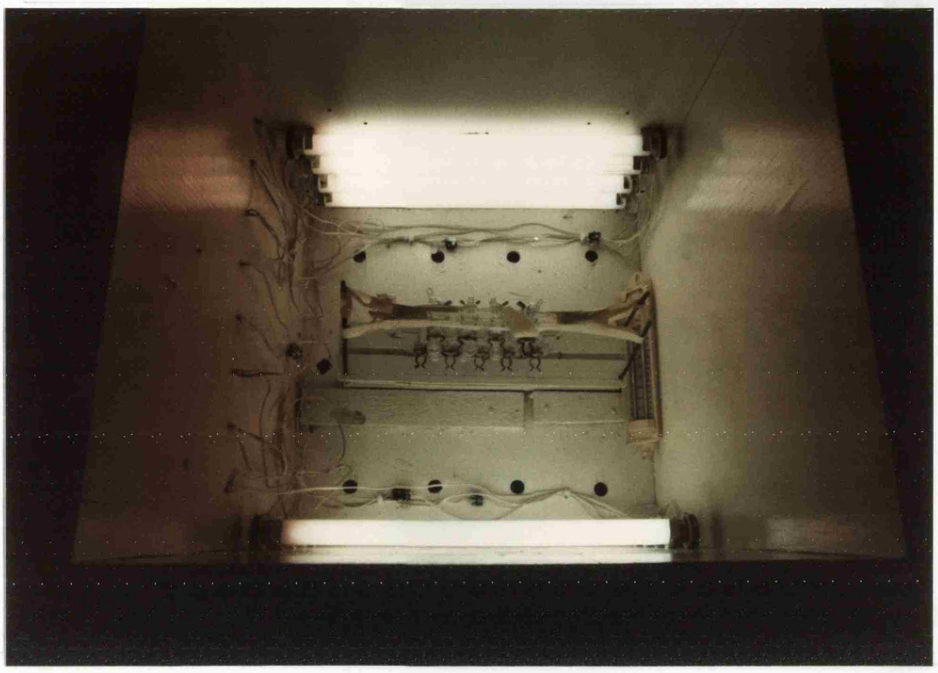


Plate 1 A typical irradiation experiment in progress.

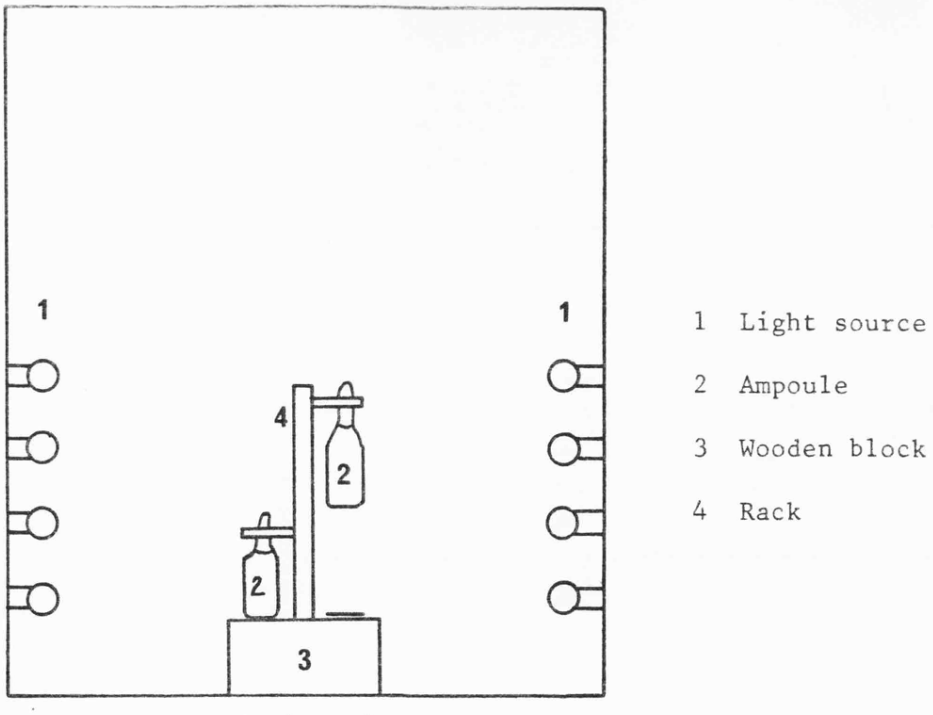


Fig.6 Diagrammatic representation of apparatus used in photochemical studies.

Irradiation Vessels

These were provided by 10ml, clear, neutral glass ampoules. A glass syringe and stainless steel needle were used to deliver 10ml of sterile thiomersal solution at a concentration of 0.008% w/v, in isotonic Sörensen's phosphate buffer (pH 7.0), into each ampoule. The ampoules were sealed and placed in foil covered beakers. It was essential that there was no delay between the preparation of the test solution and ampoules, and their transfer to the light box.

Irradiation Technique

A pair of un-irradiated ampoules was reserved for initial high-pressure liquid chromatography (HPLC) analysis. Half the remaining ampoules were then wrapped in foil and placed in the light box. These formed the heat controls and were used to monitor the effect of temperature in the light box, on unexposed thiomersal solution. The rest of the ampoules were placed in the racks for irradiation. At 2, 4, 6, 8 and 10 days, these time intervals having been determined after initial trials, four ampoules were removed from the light box, two irradiated and two heat controls. The irradiated ampoules were immediately wrapped in foil, and all four ampoules labelled and placed in foil-covered beakers at $4^{o}C$. One irradiated and one heat control ampoule were then used in microbial challenge experiments, whilst the other set were kept for HPLC analysis.

To determine the effect of storage at $4^{o}C$ and room temperature in the dark, two un-irradiated ampoules were wrapped in foil and placed in foil-covered beakers over the 10 day holding period. These were later analysed by HPLC. The effect of storing the irradiated ampoules at $4^{o}C$ for up to 10 days was also determined. The irradiation procedure

was repeated with the other buffered solutions of thiomersal, prepared to contain 0.01% EDTA and 0.1% EDTA respectively.

Analytical Methods

A reversed-phase, ion-pair HPLC system was developed in the Department for the detection of thiomersal in the presence of its photodegradation products (Appendix IV, page 286). From these studies, the following experimental conditions were found to be optimum for this separation:-

Column: 100mm x 5mm, packed with ODS - HYPERSIL

Mobile phase: CH_3CN:H_2O:CTAB(0.01M):Sörensen's Phosphate Buffer (pH5.8)

38 :40 : 12 : 10

Flow rate: 2.0ml min^{-1}

Detection range: 0.08 AUFS at 235nm

Load: 20μl

Temperature: 30°C

Instrumentation

The basic equipment is shown schematically in Figure 7 and consists of a high pressure pump, an injection port, a column and a detector. The detector signal is recorded on a chart strip recorder.

Pumping System. This was provided by a Constametric IIG (Laboratory Data Control, Division of Milton Roy), a constant flow, pulsationless solvent delivery pump. The pump is capable of delivering solvents at pressures of up to 5000 psi, at flow rates ranging from 0.1 to 10ml per minute.

Injection System. Valve injections are designed to operate at pressures in excess of 5000 psi without the use of septa. The solvent flow is by-passed into the column and the loop is filled with sample which is then introduced into the column by switching the valve around. Sample valves are the most convenient and reproducible method of sample injection. A Rheodyne, 20μl loop valve (Pye Unicam Instruments Ltd.), was

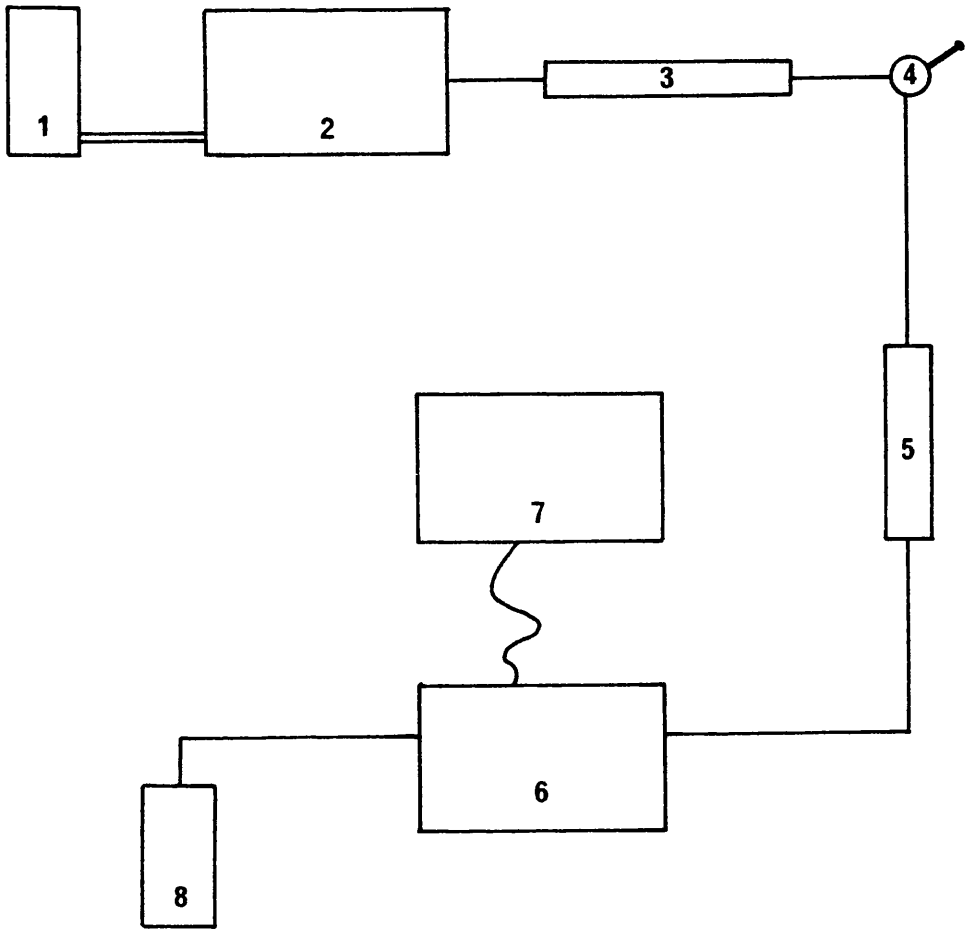


Fig.7 Typical HPLC system.

1 Mobile phase reservoir

2 High-pressure pump

3 Pre-column

4 Injection valve

5 Analytical column

6 Detector

7 Recorder

8 Waste

used to inject test samples onto the analytical column.

Analytical Column. This was provided by a 100mm x 5mm (internal dimensions), stainless steel column, (Shandon Southern Products Ltd., Cheshire). The packing material used was ODS - HYPERSIL (Shandon Southern Products Ltd.). This has a mean particle size of 5 μm. Details of the slurry packing procedure are provided later. The efficiency of the column was maintained by the use of an integral stainless steel, guard column (Shandon Southern Products Ltd.), also packed with ODS - HYPERSIL. This functioned as a trap for any particulate matter that might have escaped detection, thus preventing debris from entering the main column. The total length of the main column and guard column was 125mm. Both columns were enclosed in a copper water jacket, and fixed in position. A drawing of the water jacket is provided in Figure 8 and Plate 2 shows the jacket in position during a typical analysis.

Pre-column. The pre-column was a 150mm x 5mm, stainless steel column (Whatman Ltd.), packed with Pellosil Pellicular packing material (Whatman Ltd.). This is an absorbant, thin layer (hence pellicle), of high purity silica gel, bonded to 30-38 μm glass beads. The function of a pre-column is to reduce destruction of the packing material in the analytical column by the mobile phase.

Detecting System. A Pye Unicam LC - uv Detector (Pye Unicam Instuments Ltd.), was used to measure the varying uv absorbance of eluent from the analytical column. This is capable of detecting absorbance changes of as small as 2×10^{-5}nm, under favourable conditions.

Recording System. A potentiometric chart recorder, TE 200 series (Tekman Electronics Ltd.), was used to record the output from the detector.

Auto Sampler. Initially, samples were injected manually onto the analytical column. Towards the later stages of this work, however, an auto sampler (Applied Chromatography Systems Ltd.) was made available.

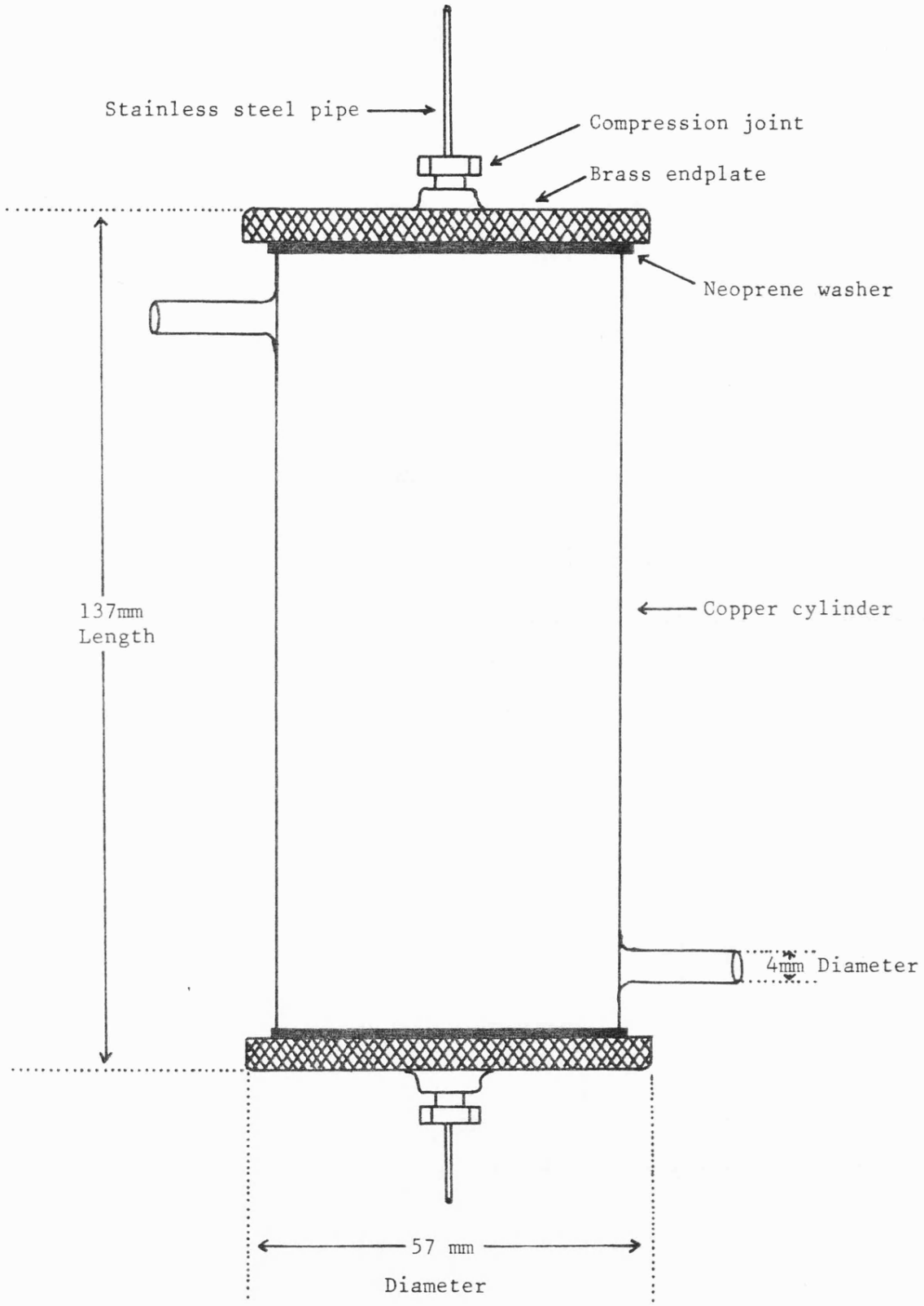


Fig.8 Water jacket for analytical column.

Plate 2 Water jacket in position during a typical analysis.

With this system, samples are presented to the injection valve via a turntable. The Rheodyne loop valve is filled under positive air pressure, so that a compressed air supply (Air Products Ltd.) is required for the operation of the valve and for transfer of the sample from the vial to the injection loop. To operate the valve correctly, a minimum air pressure of 50 psi is required.

The test solutions were pipetted into the glass vials supplied (capacity 1.5ml), which had been sprayed with black paint to prevent any further photodegradation occurring during the analysis. The caps of the vials (Chromacol Ltd., London) were then crimped into place and the samples positioned on the auto sampler turntable. A pair of vials containing standard, undegraded solutions of thiomersal were placed before each pair of degraded solutions to be analysed. Once in operation, the auto sampler analysed duplicate injections from each vial. This system has the advantage of increasing reproducibility and facilitates the handling of a larger number of samples.

Packing of Analytical Column

A CP III Slurry Packer (Jones Chromatography Ltd.), was used to pack the analytical column and its integral guard column. 2g of ODS - HYPERSIL were weighed out and transferred to a 50ml Quickfit flask. The contents were then slurried in 30ml propan-2-ol and sonicated for about two minutes to disperse any aggregates. 3ml of propan-2-ol were then pipetted into the reservoir of the packer, and the slurry transferred to this. The instructions provided with the packer were then followed and the column packed in hexane at a pressure of 4500 psi.

The packed column was disconnected, any excess material smoothed off, and the column end fittings replaced. The column was then connected

into the HPLC system, jacketed and flushed with propan-2-ol, to remove any residual hexane.

Determination of Efficiency of Analytical Column

An evaluation of the packed column was carried out next, using the test mixture for reversed-phase systems (191). A mobile phase comprising a 60:40, Methanol:Water mixture, was prepared and sonicated to remove any air bubbles present. This was allowed to circulate through the column at a flow rate of 1ml min^{-1}. The test mix components were then prepared as shown:-

1) 0.2% Phenol in 50% Methanol,

2) 0.2% Anisole in 50% Methanol,

3) 0.2% Phenetole in 50% Methanol.

The complete test mix was prepared by adding 10ml of 1), 10ml of 2), 15ml of 3), and 10ml of Methanol to a 50ml volumetric flask. The volume was then made up to 50ml with mobile phase. The chromatogram of the test mix is depicted in Figure 9. As can be seen, satisfactory resolution and peak symmetry were obtained. The 'theoretical plate number' N, was then calculated for the last peak, using the following equation:-

$$N = 5.54 \times \left[\frac{\text{retention distance}}{\text{width at } \frac{1}{2} \text{ peak height}}\right]^2 \qquad (6)$$

The value of N obtained was:-

$N = 5.54 \times (31.5)^2$, giving about 55,000 plates m^{-1}. This was considered satisfactory.

Preparation of Mobile Phase

Preparation of Counterion. The counterion used was cetyltrimethyl-

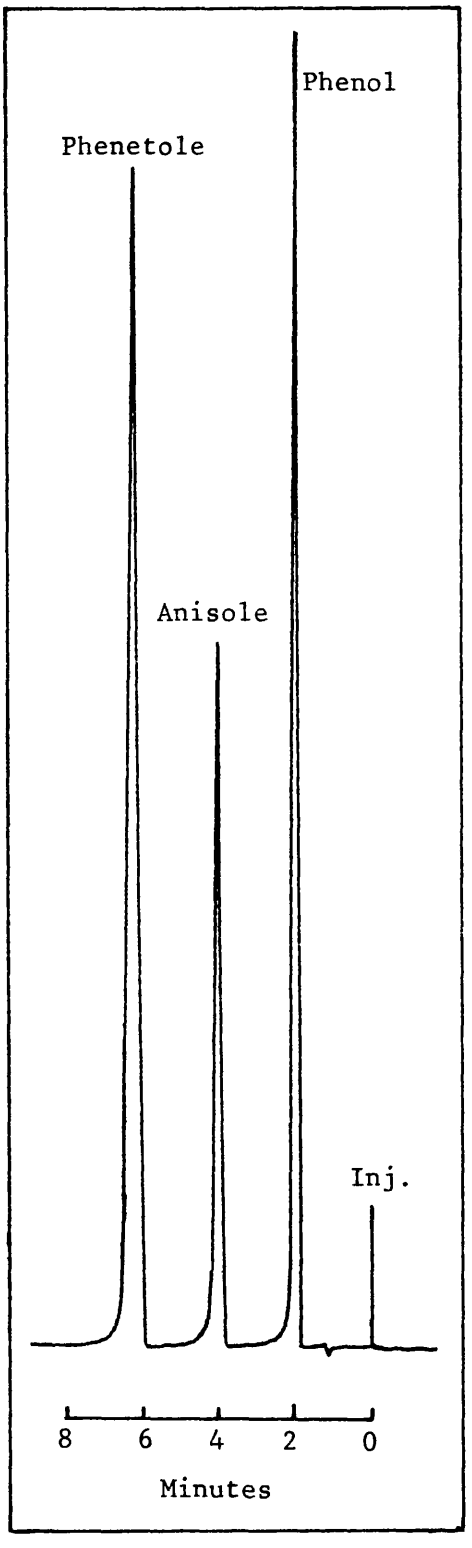


Fig.9 Chromatogram of reverse phase test solutes.

Column: 100 x 5mm, ODS-HYPERSIL
Mobile phase: Acetonitrile:Water:CTAB(0.01M):Sörensen's phosphate buffer, pH 5.8(2/15M) 38:40:12:10
Flow rate: 1.0ml per minute
Detector: uv at 254nm
Sensitivity: 0-0.32 for the first two peaks; 0-0.16 for the last peak
Load: 20 μl
Temperature: Ambient

ammonium bromide (CTAB). A 0.01M solution was prepared and maintained in a water bath at 30°C, to prevent its crystallising out with a drop in room temperature.

Preparation of Buffer. Double-strength Sörensen's phosphate buffer (pH 5.8) was prepared as indicated (185).

Preparation of Complete Mobile Phase. Since volume changes can occur during the mixing of mobile phase components, all mobile phases are prepared as volume to volume ratios. 500ml quantities were prepared in the following manner; 190ml acetonitrile were pipetted into a 500ml flask, and about 50ml water added. After mixing, the contents of the flask were allowed to return to room temperature. 50ml of the buffer were then pipetted into the flask, followed by 60ml of the CTAB solution. When the mixture was at room temperature once more, the volume was made up to 500ml with water and the mobile phase degassed in the ultrasonic bath. The mobile phase was then placed in the water bath at 30°C and the column allowed to equilibrate with re-cycling mobile phase for 1½-2 hours, before an analysis was attempted.

Care of Analytical Column

Whenever prolonged storage of the column was necessary between use, a solvent rinsing schedule was carried out. This involved flushing the column in the following order:-

1) with water, to remove any debris,
2) with methanol,
3) with 10% methanol in chloroform (the chloroform to remove any organic debris),
4) with methanol.

The column was then capped to prevent it drying out during storage. Before use, it was rinsed with water and flushed with mobile phase.

Calibration Curve for Thiomersal

To determine if there is a linear relationship between the concentrations of thiomersal and the equivalent peak heights, it was necessary to do a calibration curve plot. This involved preparing a series of dilutions of a stock thiomersal solution in isotonic Sörensen's phosphate buffer (pH 7.0), to give concentrations of between 0.002% and 0.01% w/v. These were then diluted in a 66% acetonitrile in buffer (pH 5.8) solution in a 40:60 ratio. Five injections of each concentration were then made onto the column, and the resulting peak heights measured. The data together with a statistical analysis are shown in Table 18. A graph of mean peak height against concentration of thiomersal is presented in Figure 10.

As column performance can vary with prolonged use, the slope of the calibration curve cannot always be used with confidence, to determine the concentration of thiomersal. In practice, therefore, duplicate standard solutions of a known thiomersal concentration were injected to 'bracket' injections of the test sample. In this way, the concentration of thiomersal in the test solution could be calculated with reference to the standard solution.

TABLE 18

Effect of varying thiomersal concentration on peak heights.

Test thiomersal solutions were prepared in isotonic Sörensen's phosphate buffer (pH 7.0). Each peak height shown is the mean of 5 injections.

Thiomersal Concentration (% w/v)	Mean Peak Height‡‡ (mm)	Standard Deviation	Coefficient of Variation (%)
0.002	26.06	0.364	1.40
0.004	55.72	1.173	2.10
0.006	84.40	1.194	1.41
0.008	115.54	2.475	2.14
0.010	142.00	2.806	1.98

‡‡After dilution of the test sample in 66% acetonitrile in pH 5.8 phosphate buffer, (40:60).

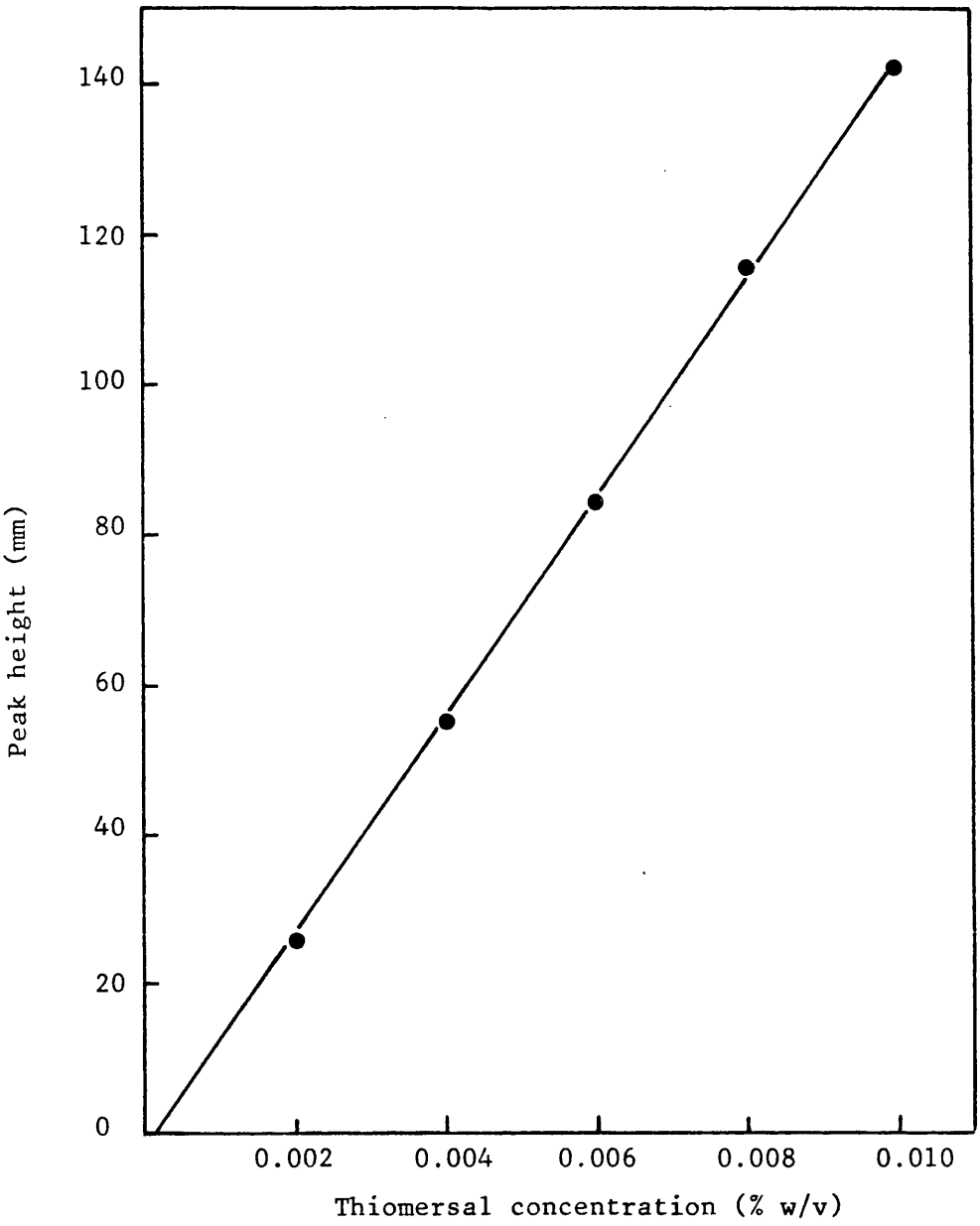


Fig.10 Calibration curve for thiomersal.

EXPERIMENTAL

MICROBIOLOGICAL STUDIES

GROWTH CHARACTERISTICS OF TEST MICRO-ORGANISMS

The growth of each test organism in the appropriate chemically defined medium was followed by viable count determinations and optical density readings. Samples were removed hourly up to 8 hours and thereafter at two-hourly intervals up to 26 hours for plate counts; optical density measurements were made on a two-hourly basis throughout. Growth in TSB was also followed for a selected Gram-positive (Staph. aureus), and a Gram-negative organism (Ps. aeruginosa). The results of these experiments are presented in Figures 11-15.

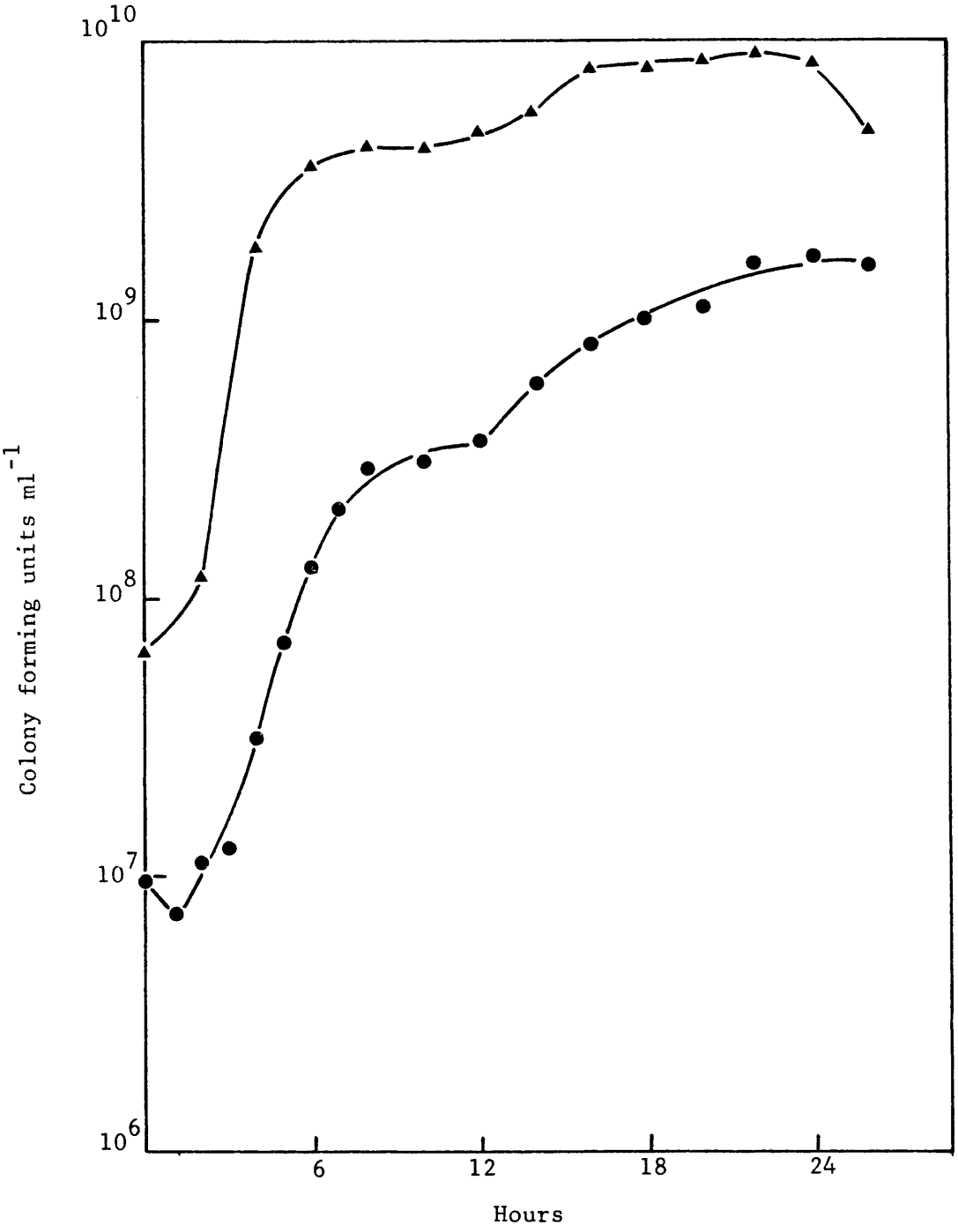


Fig.11 Growth curves obtained for Staph. aureus in defined medium and TSB, using the viable count method.

● Growth in defined medium

▲ Growth in TSB

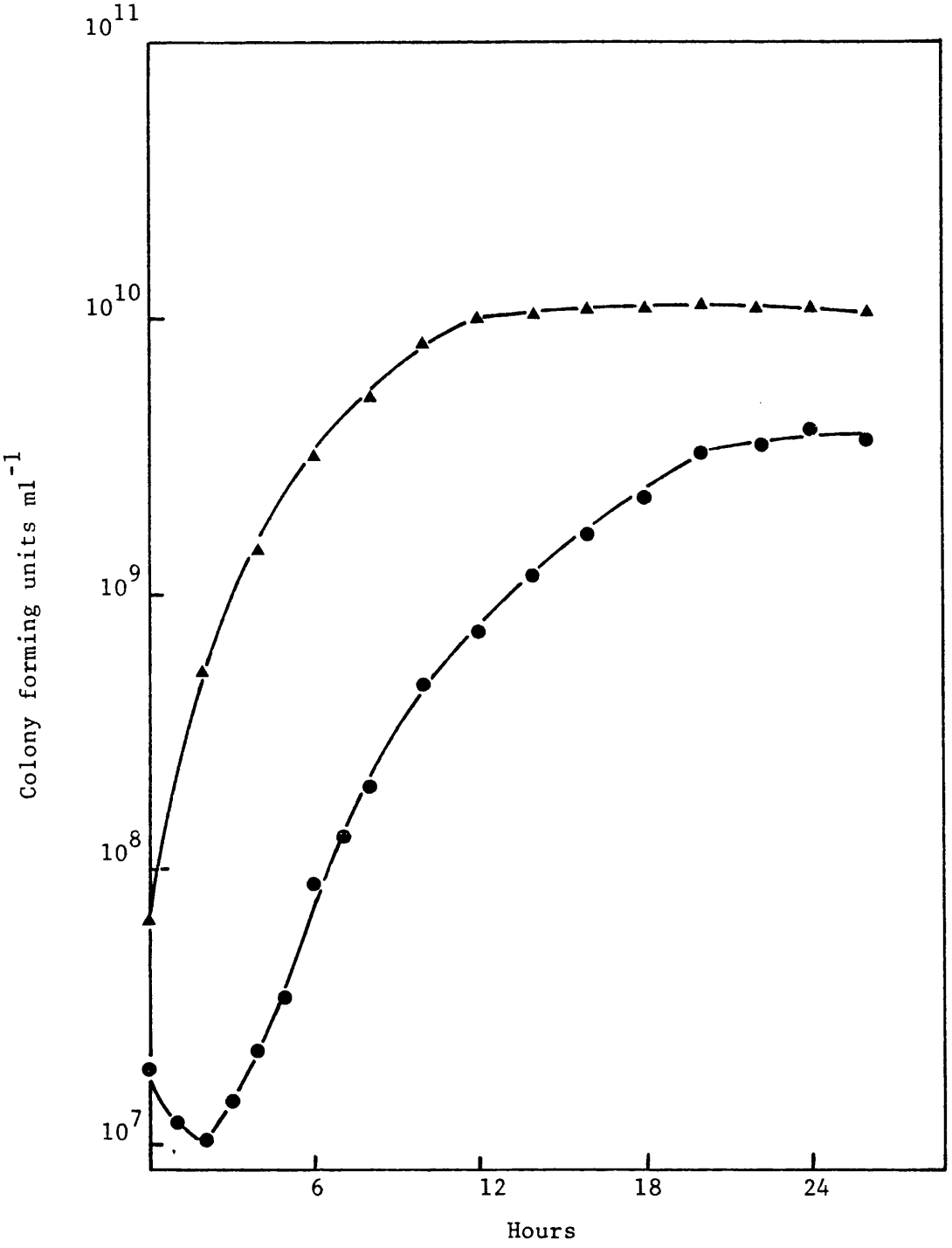


Fig.12 Growth curves obtained for Ps. aeruginosa in defined medium and TSB, using the viable count method.

● Growth in defined medium

▲ Growth in TSB

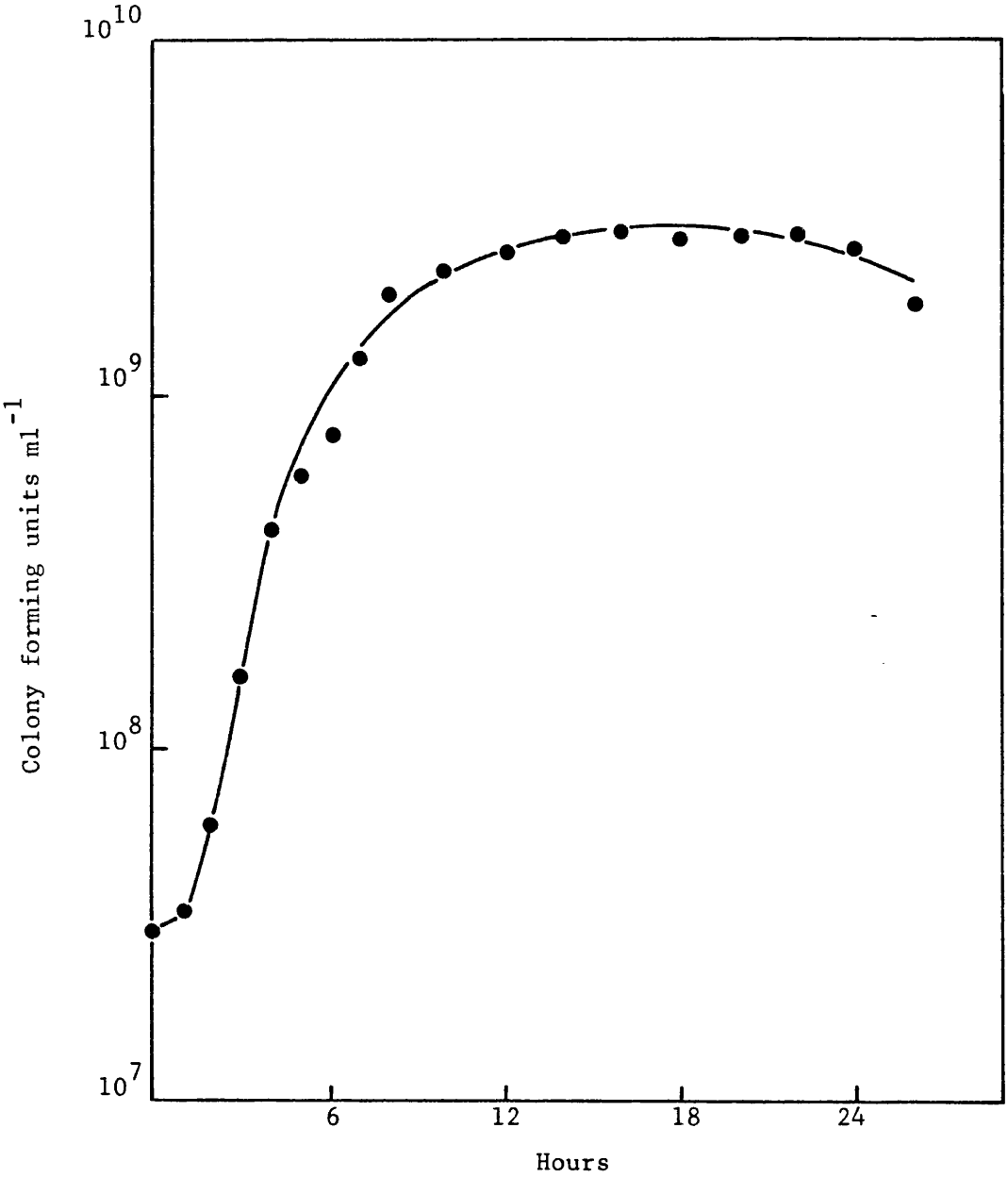


Fig.13 Growth curve obtained for E. coli in defined medium, using the viable count method.

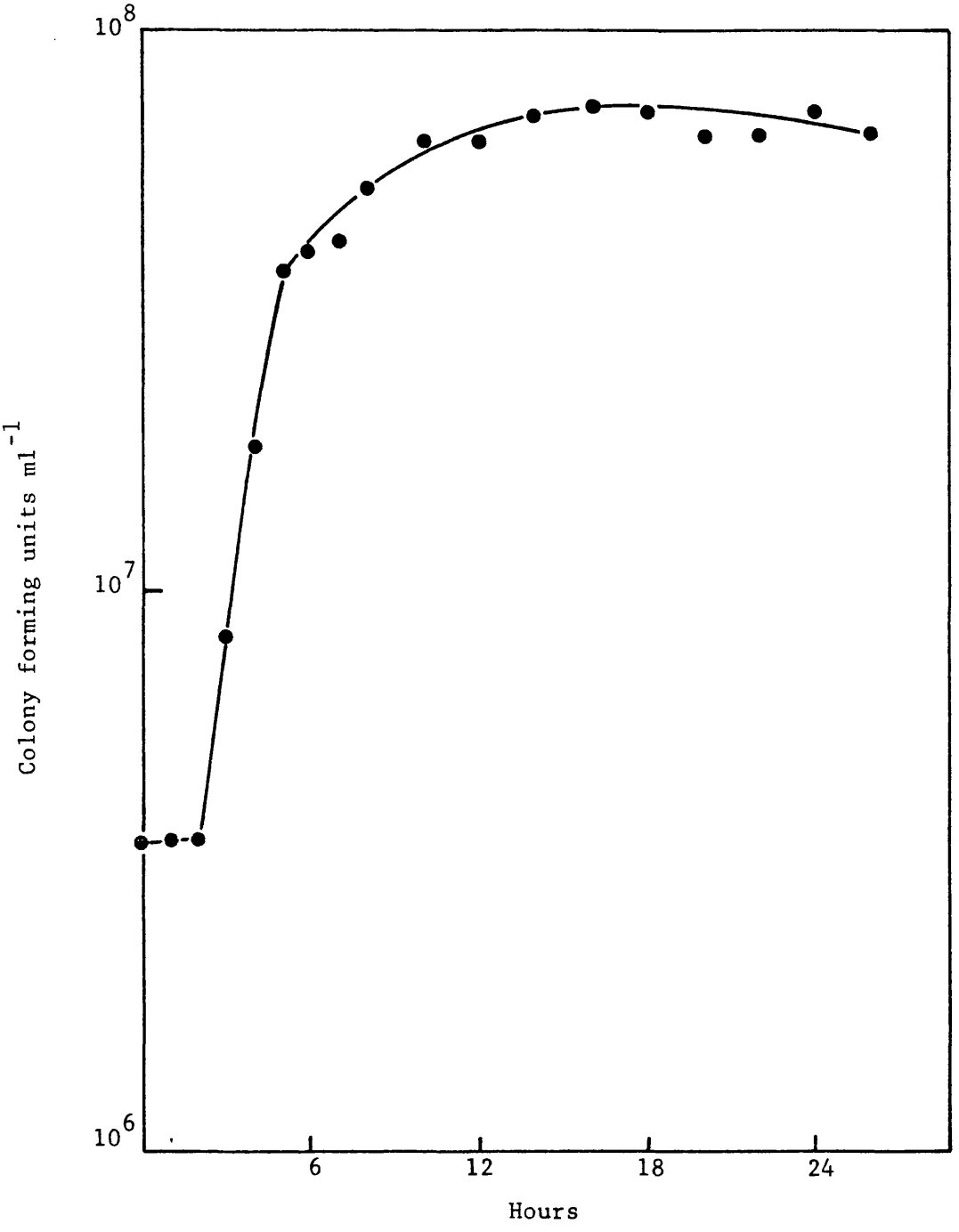


Fig.14 Growth curve obtained for C. albicans in defined medium, using the viable count method.

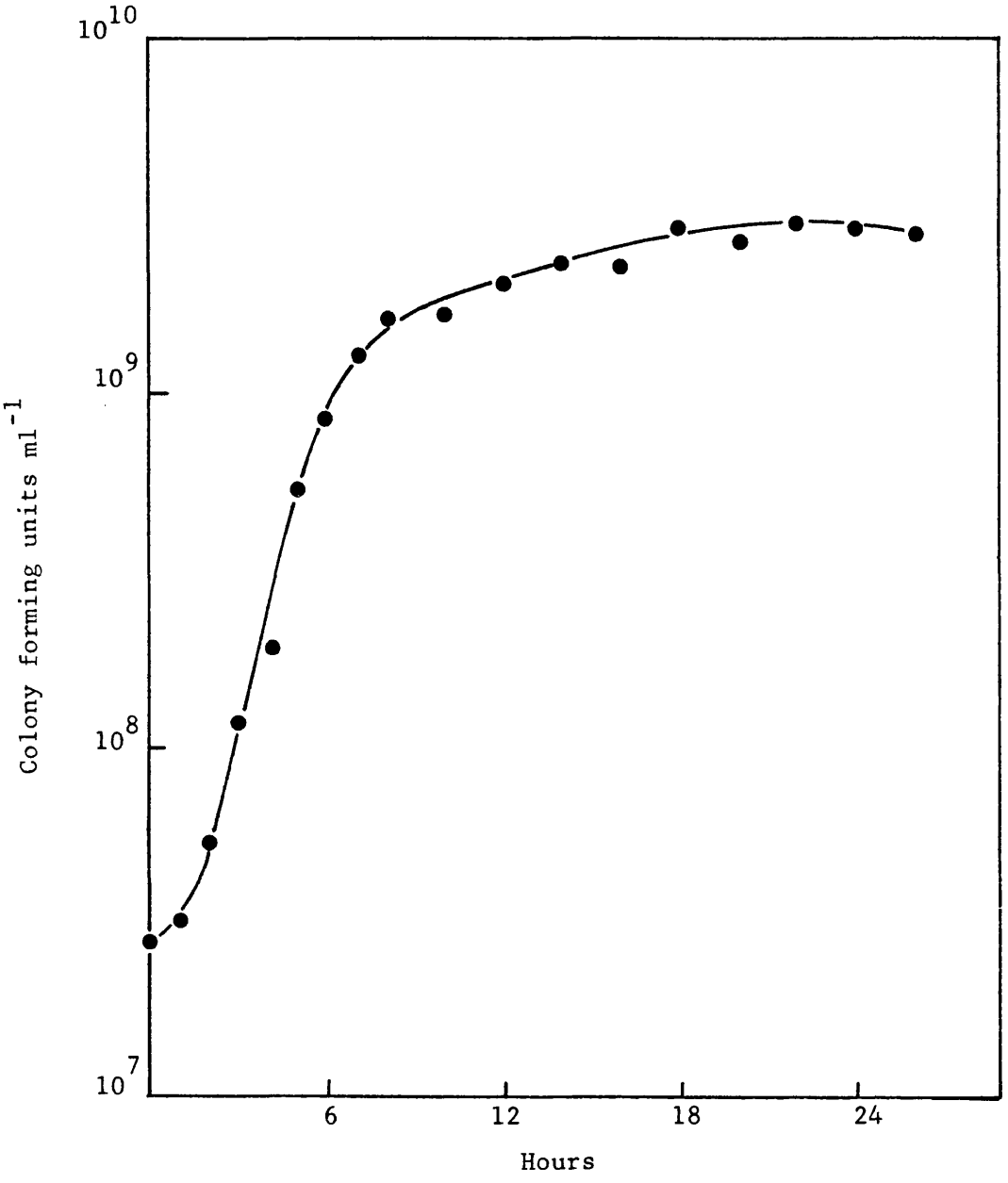


Fig.15 Growth curve obtained for B. subtilis in defined medium, using the viable count method.

EFFECT OF EXPERIMENTAL CONDITIONS ON THE ANTIMICROBIAL ACTIVITY OF THIOMERSAL

1) Effect of Concentration

Four concentrations of thiomersal were selected for testing, to cover the range over which it is normally used as a preservative. These were 0.001%, 0.004%, 0.008% and 0.01% w/v. Suitable dilutions in isotonic Sörensen's phosphate buffer (pH 7.0) were made from a freshly-prepared 0.1% w/v stock solution of thiomersal, and the standard challenge technique followed.

Figures 16-20 depict the results of these experiments and Tables 19 and 20 present the $t_{0.1}$ values recorded and the concentration exponents calculated for each test organism respectively. The survival of a test organism over the duration of an experiment has only been plotted in all figures where changes in numbers of survivors occurred to a noticeable extent. In all graphs throughout this work, a - - -→ at the end of a plot indicates that, at the next sampling point, no survivors were found.

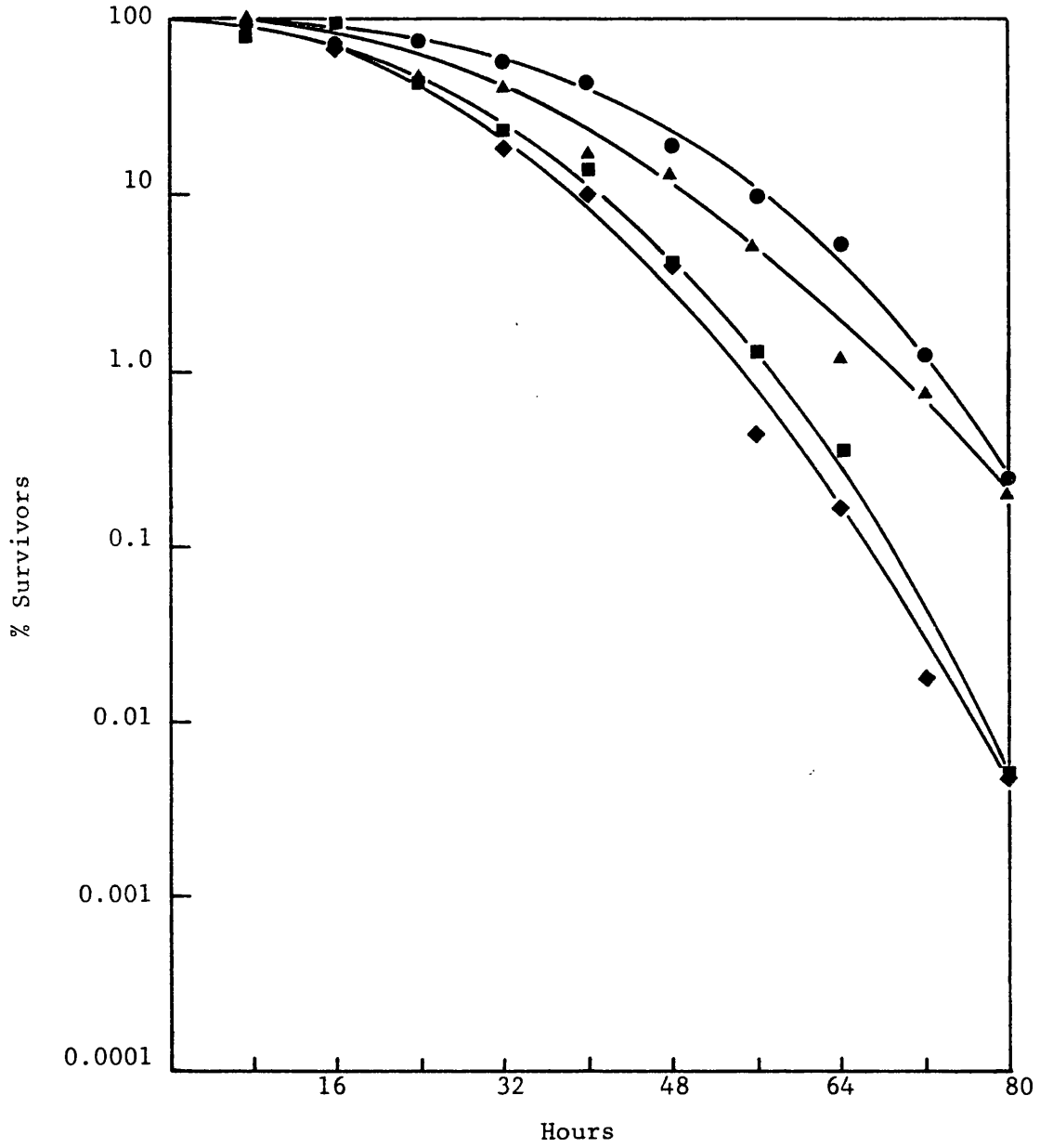


Fig.16 Effect of 0.001% (●), 0.004% (▲), 0.008% (■), and 0.01% (◆) w/v thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0, at 25°C on Staph. aureus.

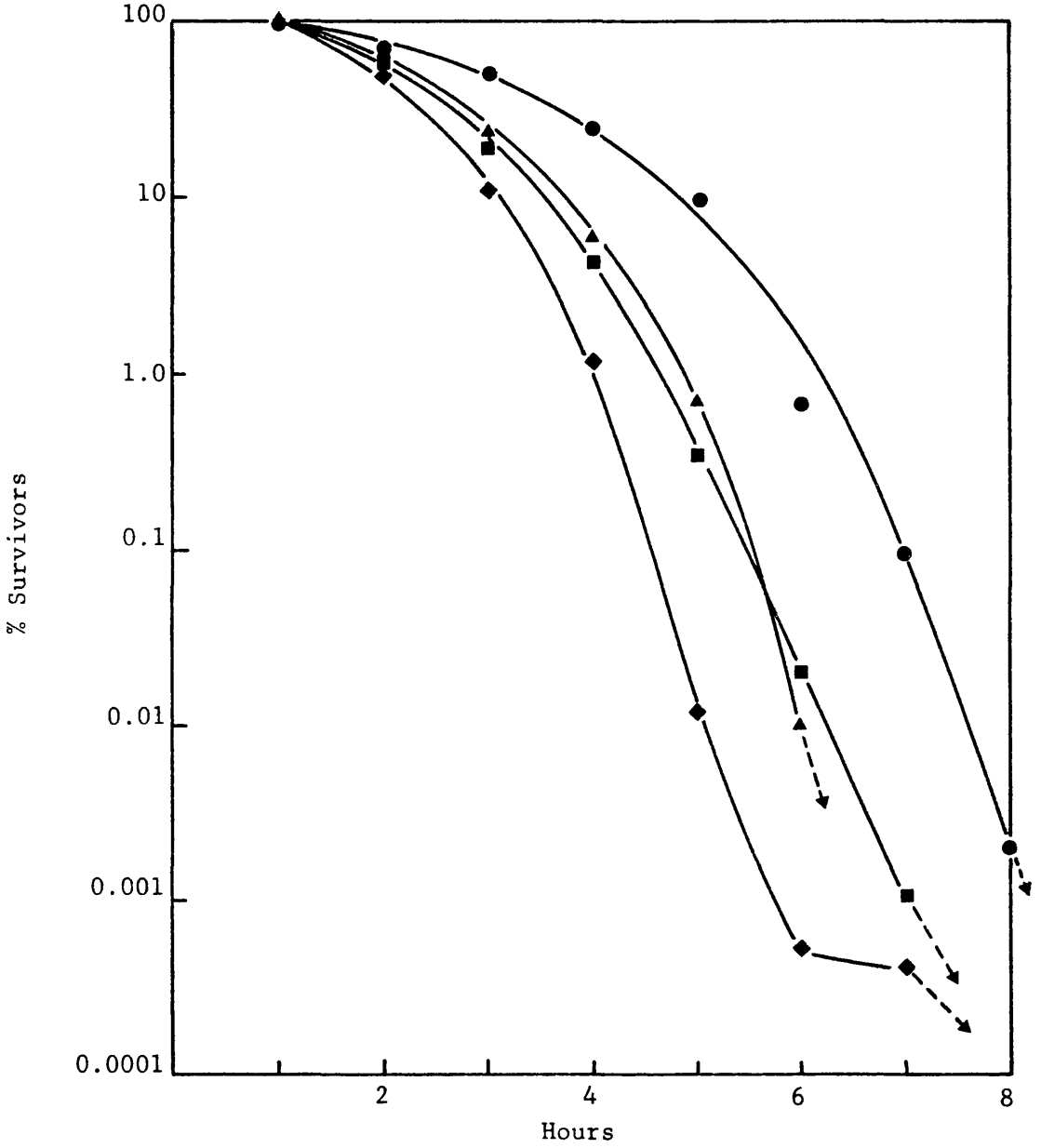


Fig.17 Effect of 0.001% (●), 0.004% (▲), 0.008% (■), and 0.01% (◆) w/v thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0, at 25°C on Ps. aeruginosa.

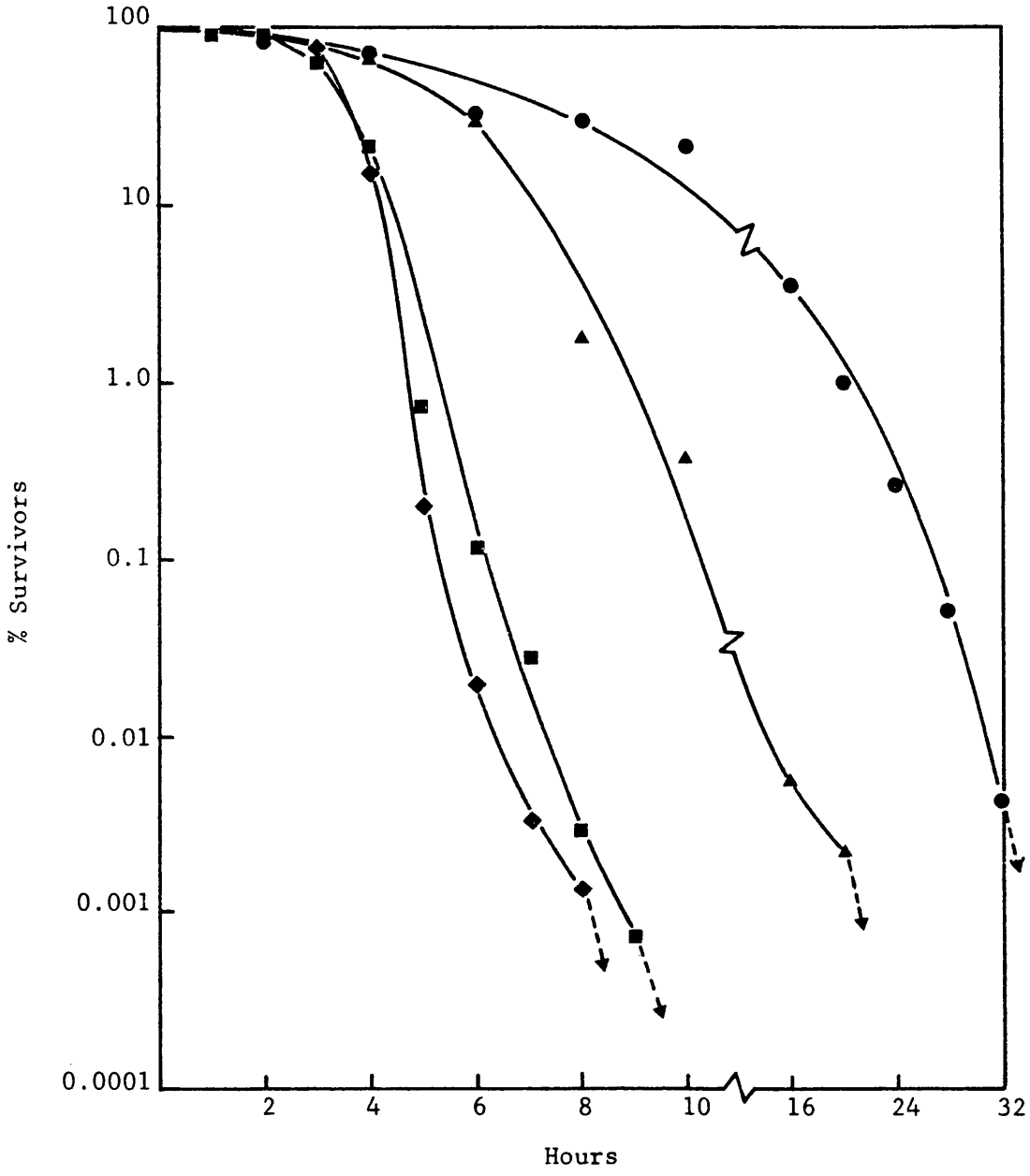


Fig.18 Effect of 0.001% (●), 0.004% (▲), 0.008% (■) and 0.01% (◆) w/v thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0, at 25°C on E. coli.

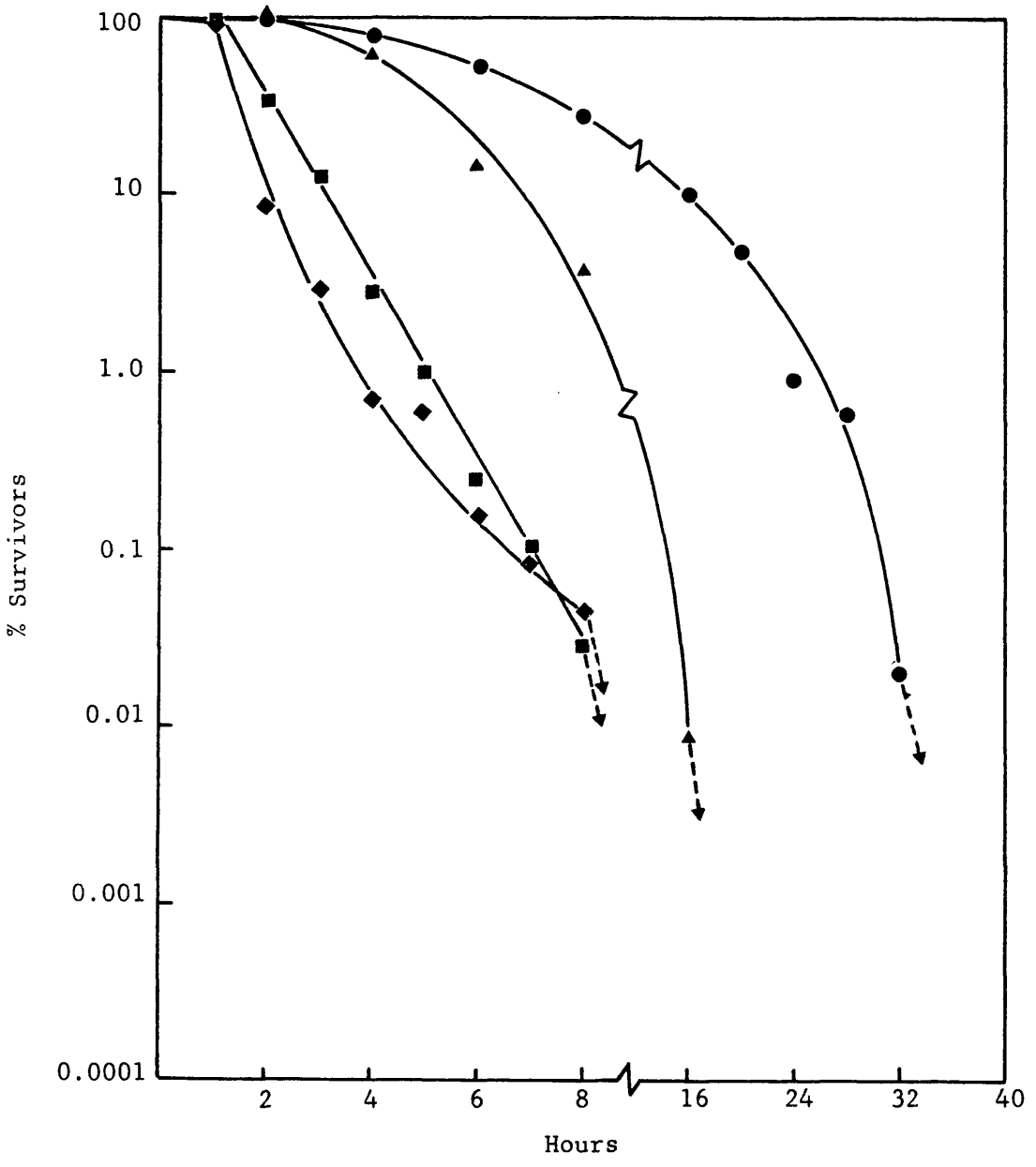


Fig.19 Effect of 0.001% (●), 0.004% (▲), 0.008% (■) and 0.01% (◆) w/v thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0, at 25°C on C. albicans.

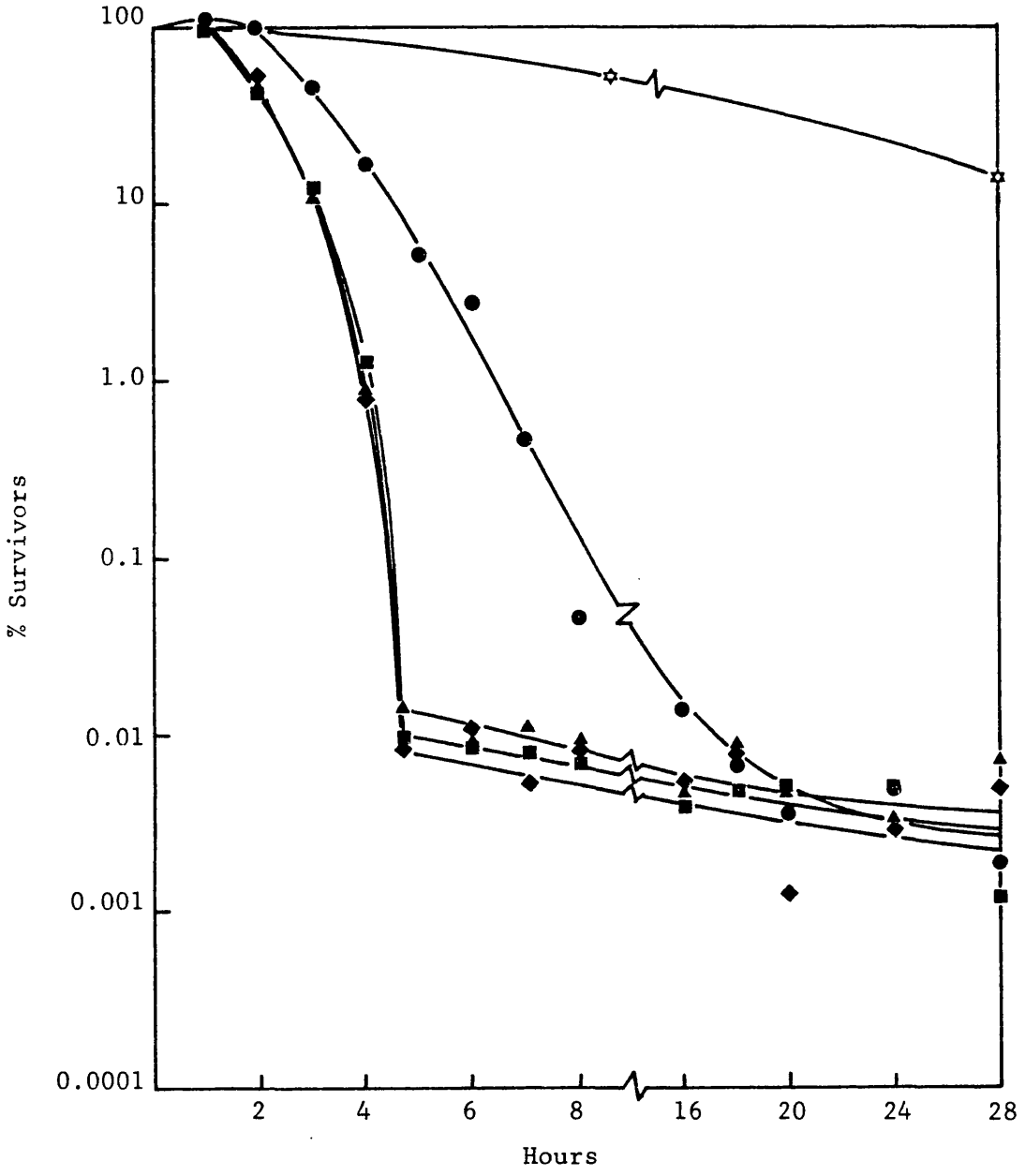


Fig.20 Effect of 0.001% (●), 0.004% (▲), 0.008% (■) and 0.01% (◆) w/v thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0, at 25°C on B. subtilis.

(✡) survival in buffer only

TABLE 19

‡‡$t_{0.1}$ values recorded from the effect of concentration on the antimicrobial activity of Thiomersal.

Concentration of Thiomersal (%, w/v)	$t_{0.1}$ values (hours) ‡‡Staph. aureus	Ps. aeruginosa	E. coli	C. albicans	B. subtilis
0.001	73.5	6.75	26.1	29.75	8.0
0.004	68.0	5.60	10.0-16.0	8.0-16.0	4.4
0.008	57.5	5.50	6.1	7.1	4.6
0.01	54.0	4.55	5.3	6.6	4.4

‡‡$t_{1.0}$ values were read for Staph. aureus.

TABLE 20

Concentration Exponents calculated for the Test Organisms.

Test Organism	‡Concentration Exponent (n)	Average value of n
Staph. aureus	0.13	
Ps. aeruginosa	0.17	
E. coli	0.69	0.38
C. albicans	0.65	
B. subtilis	0.26	

‡Calculated by substitution in equation 2, page 5 , using the $t_{0.1}$ values ($t_{1.0}$ values for Staph. aureus), obtained with 0.001% and 0.01% w/v thiomersal from Table 19.

Reproducibility of Survival Curves. From the results of the previous experiment, 0.008% w/v thiomersal was selected as the standard concentration to be used in all subsequent experiments. This concentration, whilst lying within the range of concentrations in which thiomersal is normally used, has the advantage of bringing about a drop in viable count of three log cycles within a working day, for all the test organisms apart from Staph. aureus.

To measure how reproducible the experimental protocol was in determining the antimicrobial activity of thiomersal, the experiment was repeated a further five times, using a freshly-prepared test solution in each case, and five separate cultures of each test organism. The results of these experiments are depicted in Figures 21-25, where the mean per cent survivors and standard deviation have been plotted. Table 21 lists the $t_{0.1}$ values obtained from these curves and Table 22 provides a brief description of the colonial morphology of the surviving organisms, together with any changes noted.

The plots obtained as a result of these experiments have been represented in all subsequent figures by a dashed line (---), and depict the effect of the standard test solution on each challenge organism. A standard test solution was always included for each experiment, however, and a limited number of samples taken from this to ensure that the response of the organisms had not changed.

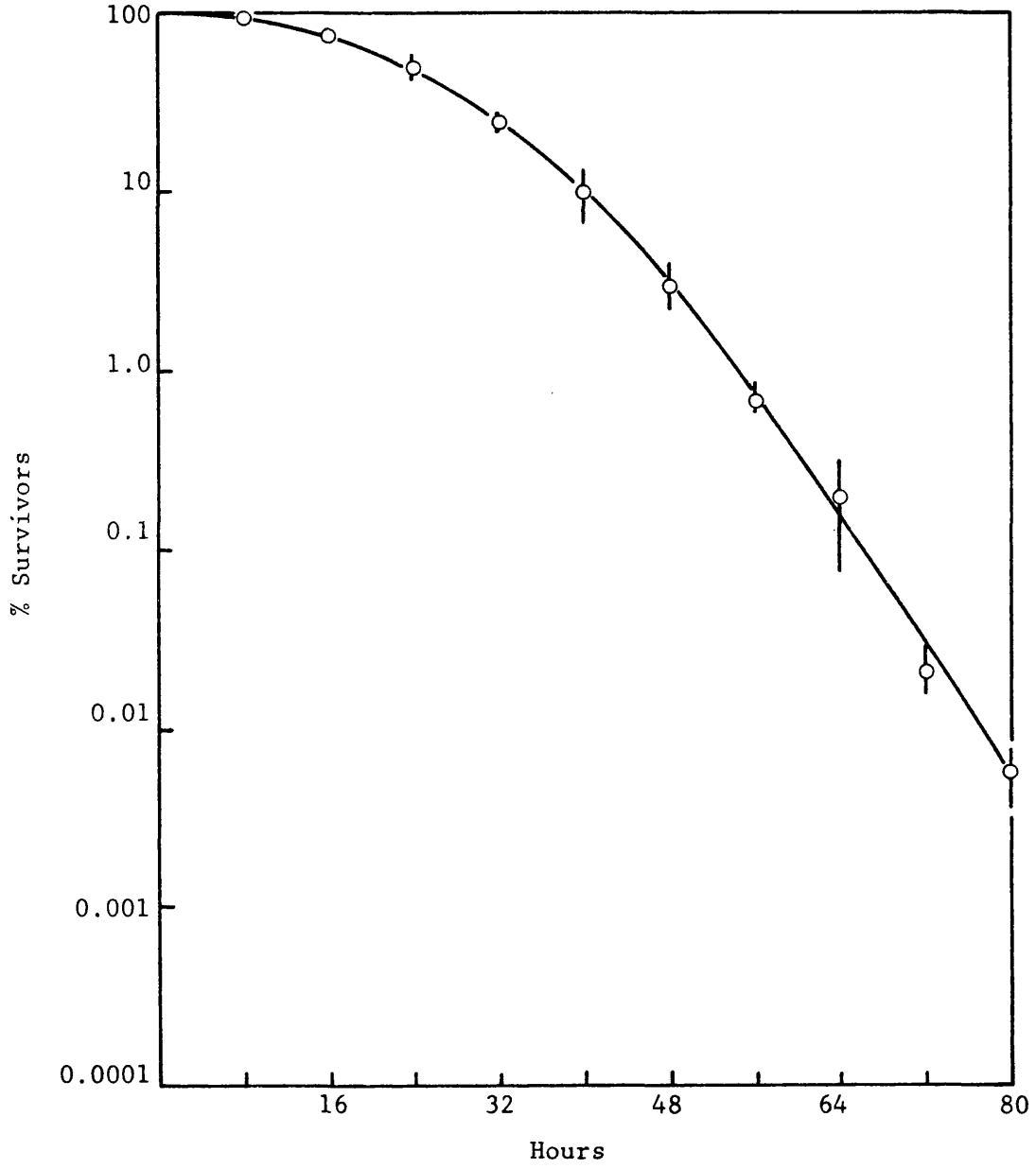


Fig.21 Effect of 0.008% w/v thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0, at 25°C on Staph. aureus. (Each point is the mean of five experiments).

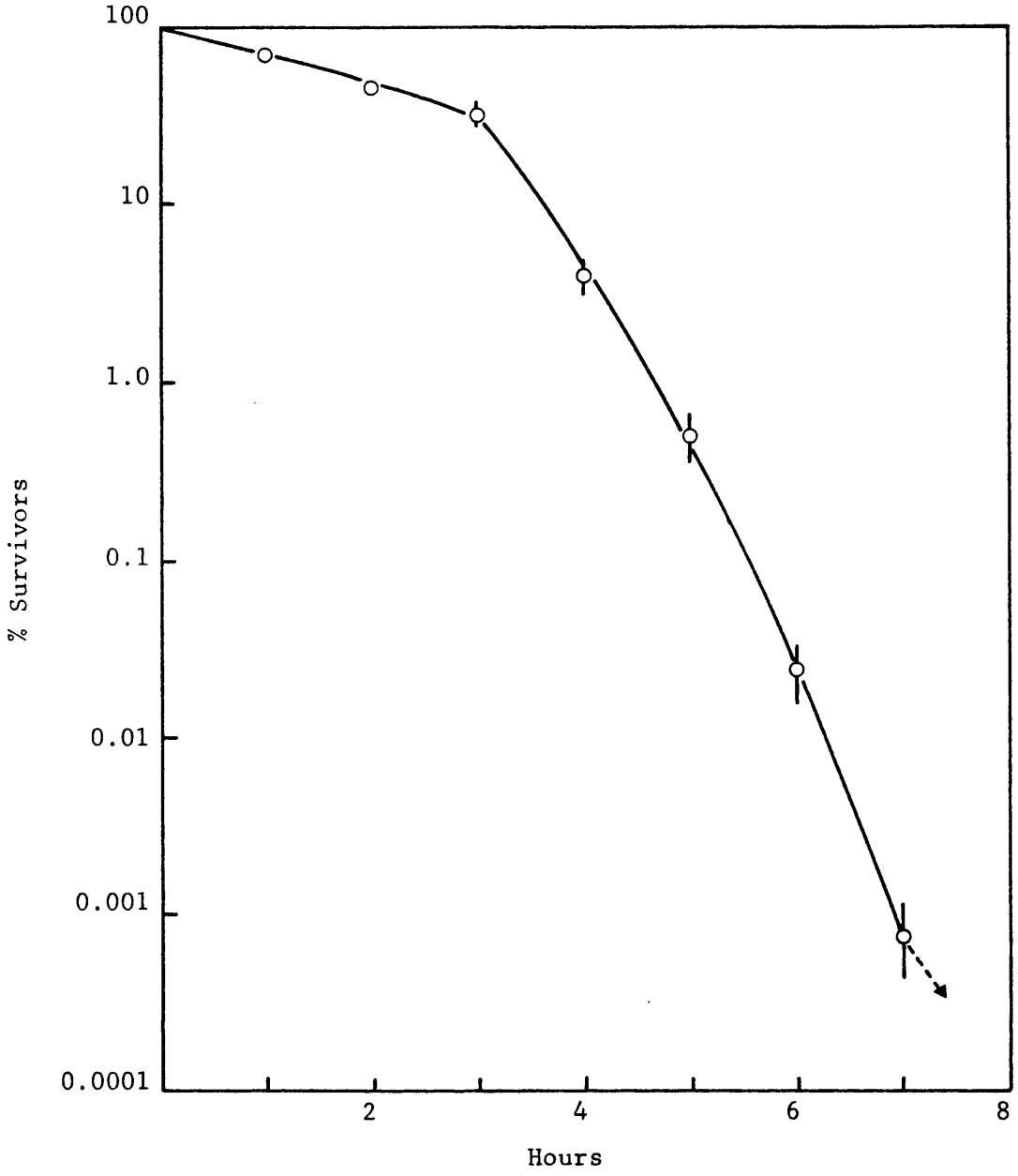


Fig.22 Effect of 0.008% w/v thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0, at 25°C on Ps. aeruginosa. (Each point is the mean of five experiments).

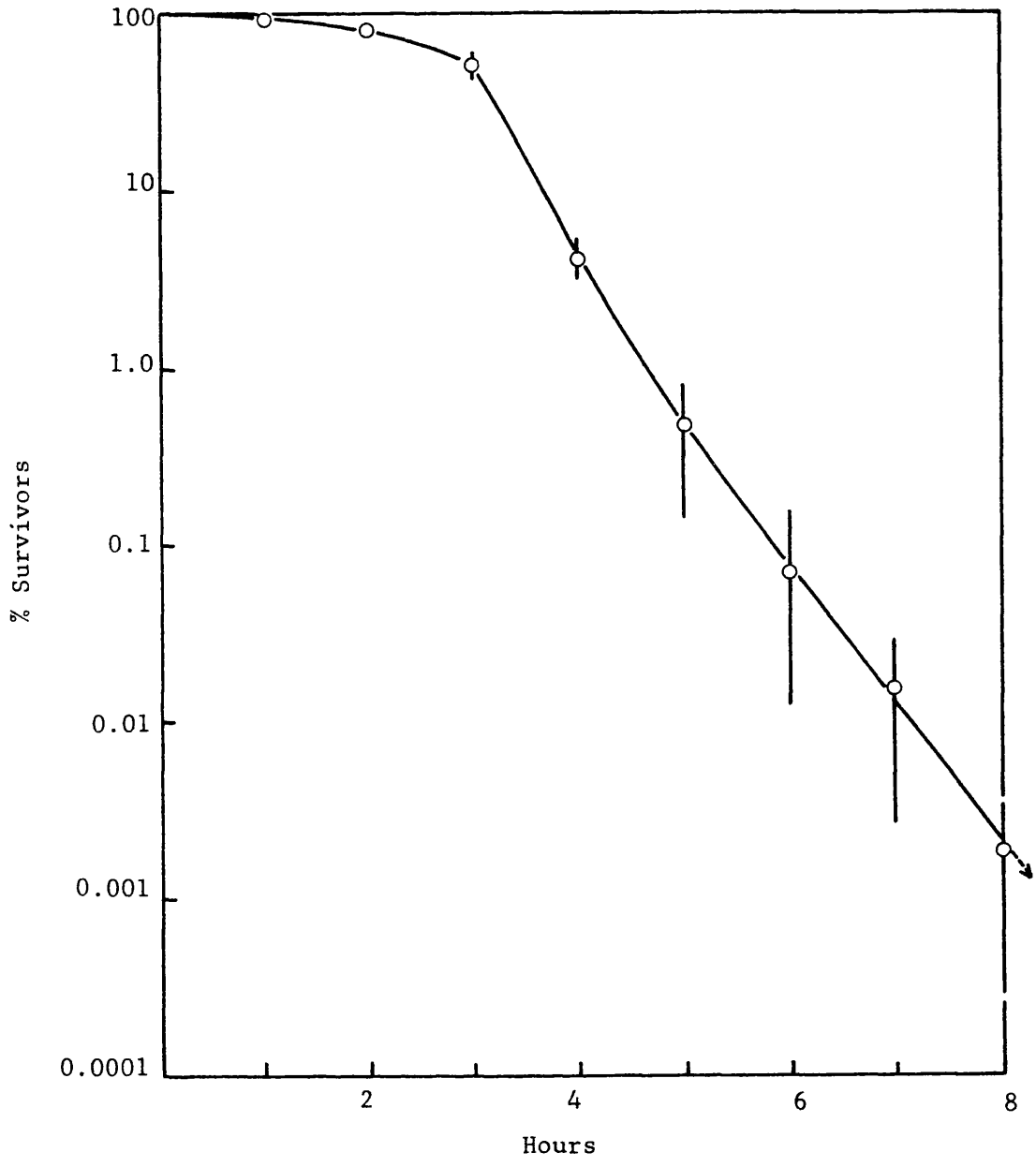


Fig.23 Effect of 0.008% w/v thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0, at 25°C on E. coli. (Each point is the mean of five experiments).

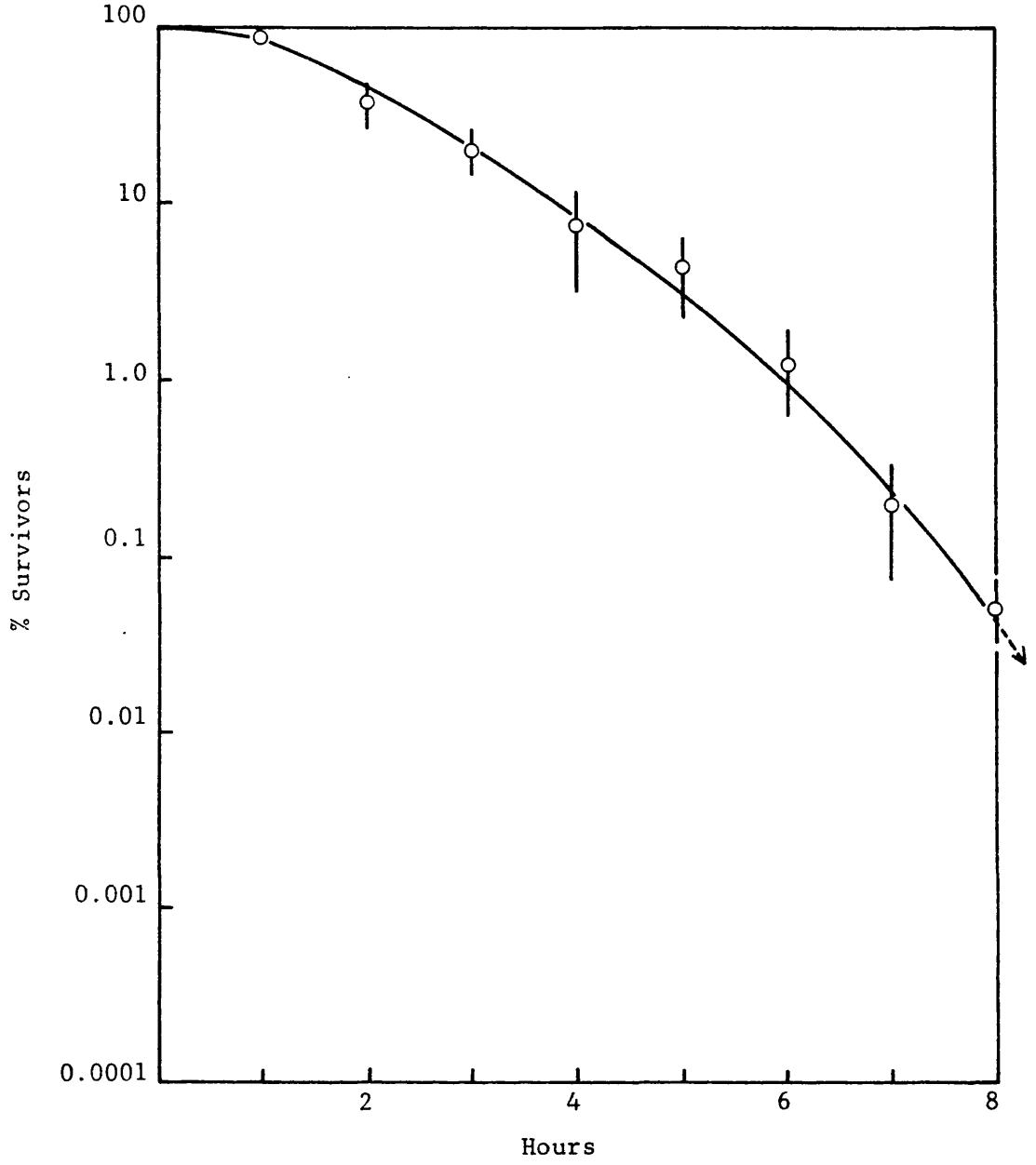


Fig.24 Effect of 0.008% w/v thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0, at 25°C on C. albicans. (Each point is the mean of five experiments).

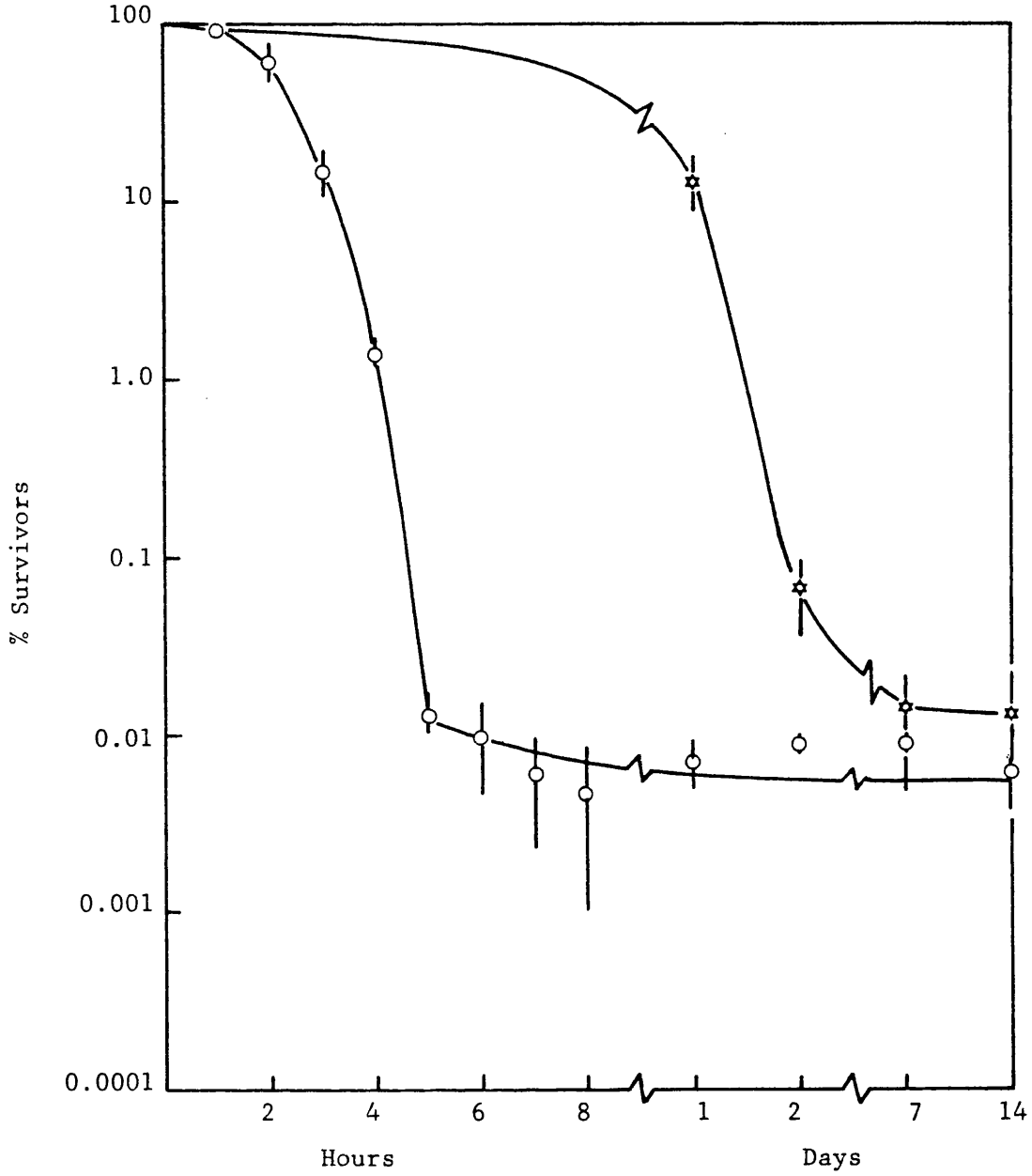


Fig.25 Effect of 0.008% w/v thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0, at 25°C on B. subtilis. (Each point is the mean of five experiments).

(✡) survival in buffer only

TABLE 21

$t_{0.1}$ values recorded from the mean effect of 0.008% w/v thiomersal on the test organisms.

Test Organism	$t_{0.1}$ values (hours)
Staph. aureus	66.5
Ps. aeruginosa	5.55
E. coli	5.95
C. albicans	7.55
B. subtilis	4.4

TABLE 22

Colonial Morphology of Surviving Organisms.

Temperature of Experiment: 25°C

Incubation Conditions : 48 hours at 37°C

(24 hours at 37°C for B. subtilis)

Plating Medium : TSA (Lab M)

Test Organism	Colonial Morphology
Staph. aureus NCTC 6571	After 24 hours in the test solution, a small number of colonies which were reduced in size (< 1mm), appeared on plates from this sample. These continued to appear in subsequent samples, ranging initially from about 2% of the total count and reaching a maximum of about 10% from the 40 hour sample. After this, they formed between 3-6% of the colonies, but were not found on the 72 or 80 hour plates. Increased incubation did not produce an increase in size of these colonies and the effect was not apparent in colonies plated from the control tube, where the staphylococci had been in buffer only.
Ps. aeruginosa NCTC 6750	Plates from the 3 hour sample showed reduced pigmentation in the medium and colonies were now a yellow-green, where originally they had been a deep green. The 6 hour sample plates had very little pigment present in the medium and colonies were a pale yellow-green. Plates

Test Organism	Colonial Morphology
Ps. aeruginosa cont'd	from the control tube showed pigment in their medium, but colonies here had also lost their deep green colour and were now yellow green.
E. coli NCTC 86	Any effect on morphology is very slight. A small increase in colony size was apparent in a few colonies (from 3mm to 4-5mm), after the 3 hour sample. This was not found with colonies from the control tube.
C. albicans LSHTM 3153	The effect seen here is similar to that observed with Staph. aureus. At the 1 hour sample, a few colonies (about 7%), were reduced in size (< 1mm); the proportion of these increased with the 2, 3 and 4 hour samples, until at the latter, they represented about 20% of the survivors. No such effect was apparent with the 5 hour sample or in subsequent samples. Plates from the control tubes did not show this effect.
B. subtilis FD TEMP	No effect was seen here until the 4 hour sample. Colonies from the '0' hour sample were 4-6mm in diameter and had a 'frill' around the edges. The colonies that showed an effect at 4 hours were reduced in size (3-5mm) and had a very reduced 'frill', or none at all. The effect disappeared in later samples and was not found in colonies from the control tube.

2) Effect of Plating Media

The composition of plating medium has been reported to affect the recovery of 'damaged' micro-organisms, so it was decided to determine the effect of other commercially available media on the recovery of such organisms. For this purpose, Columbia Agar Base (CAB) and Nutrient Agar (NA), were selected for bacterial challenge experiments, and CAB and Malt Extract Agar (MEA), were chosen for experiments with C. albicans Table 23 lists the consituents of the media used. CAB will not support the growth of fastidious micro-organisms if used unsupplemented; NA and TSA are both general purpose laboratory media; MEA is recommended for the cultivation of saprophytic and parasitic yeasts and fungi.

The results of these experiments are depicted in Figures 26-30, and brief descriptions of colonial morphology are outlined in Tables 24a-e. The $t_{0.1}$ values obtained from the challenge experiments are recorded in Table 25. On the basis of these findings, it was decided to continue using TSA as the plating medium.

TABLE 23

Composition of Commercial Media used.(g l^{-1}).

Constituents	CAB (Lab M)	MEA (Oxoid)	NA (Oxoid)	TSA (Lab M)
'Lab M' Columbia-peptone Mixture	23			
Mycological Oxoid peptone (L40)		5		
Peptone Oxoid (L37)			5	
Soy Peptone				5
Tryptone				15
'Lab-Lemco' Powder Oxoid (L29)			1	
Corn Starch	1			
Malt Extract Oxoid (L39)		30		
Yeast Extract Oxoid (L20)			2	
Sodium Chloride	5		5	5
Agar No. 1 Oxoid (L11)		15		
Agar No. 3 Oxoid (L13)			15	
'Lab M' Agar	15			
'Lab M' Agar No. 2				12

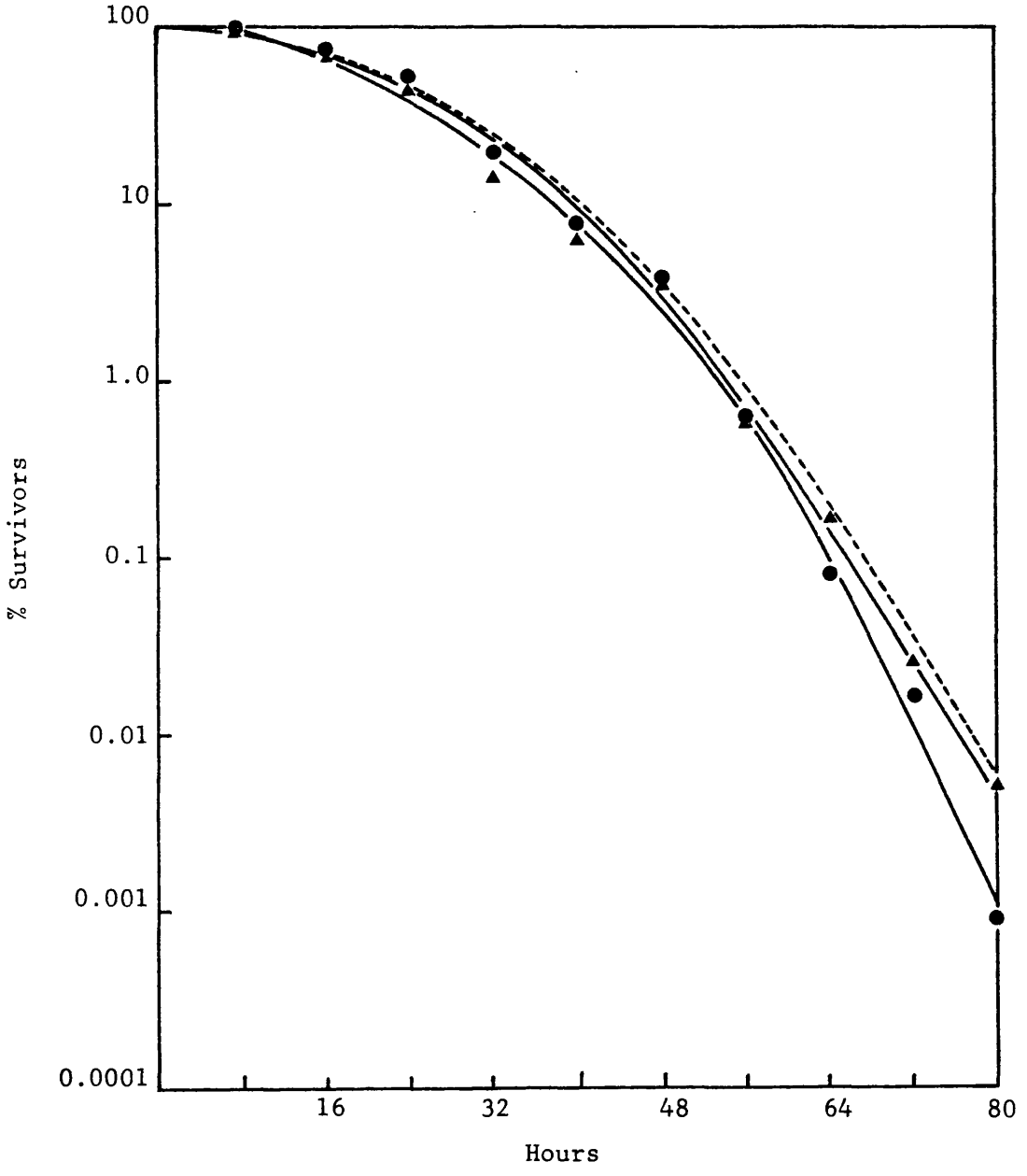


Fig.26 The recovery of Staph. aureus on Columbia Agar Base (●), Nutrient Agar (▲) and Tryptone Soya Agar (---), after treatment with 0.008% w/v thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0, at 25°C.

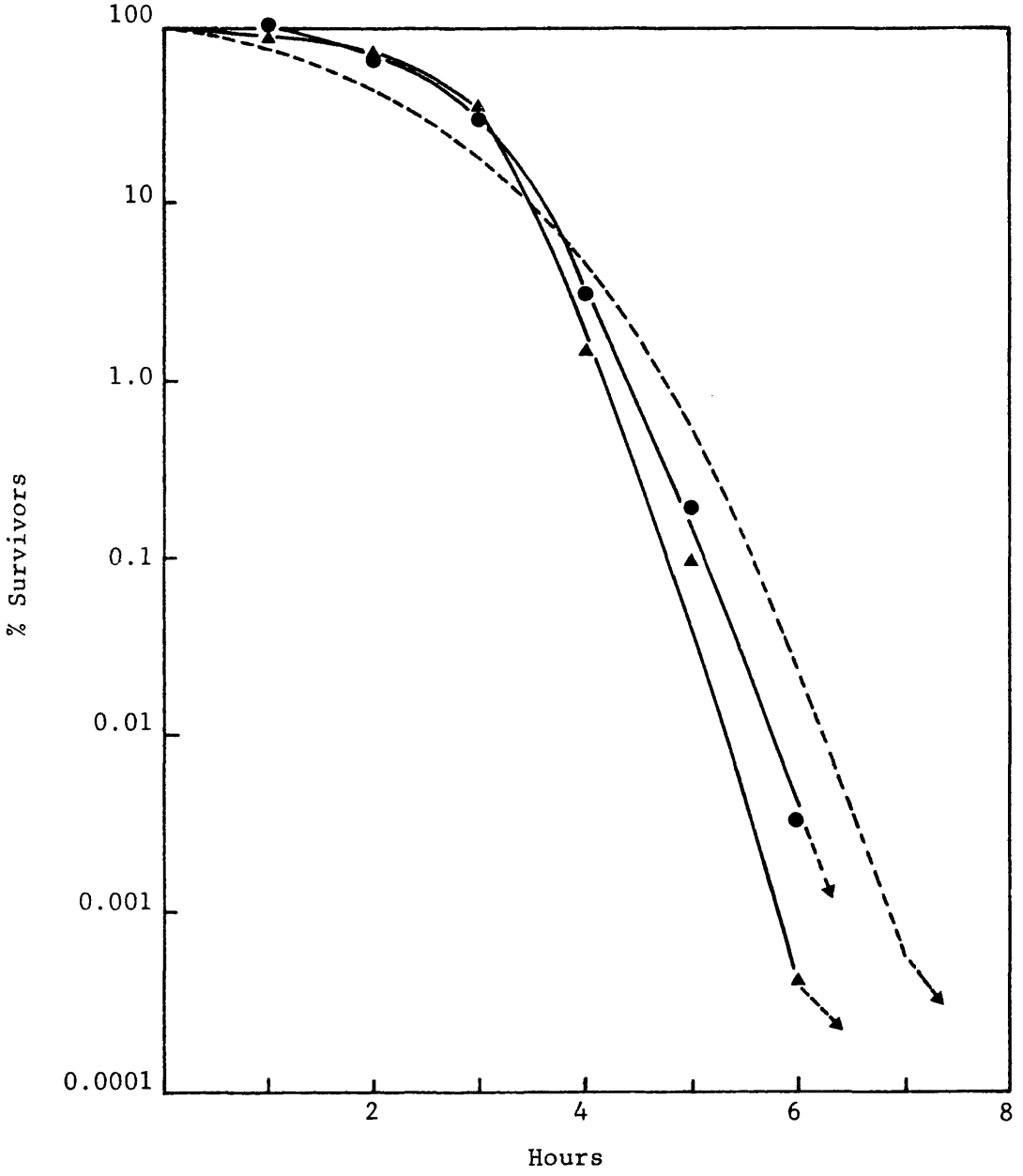


Fig.27 The recovery of Ps. aeruginosa on Columbia Agar Base (●), Nutrient Agar (▲) and Tryptone Soya Agar (---), after treatment with 0.008% w/v thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0, at 25°C.

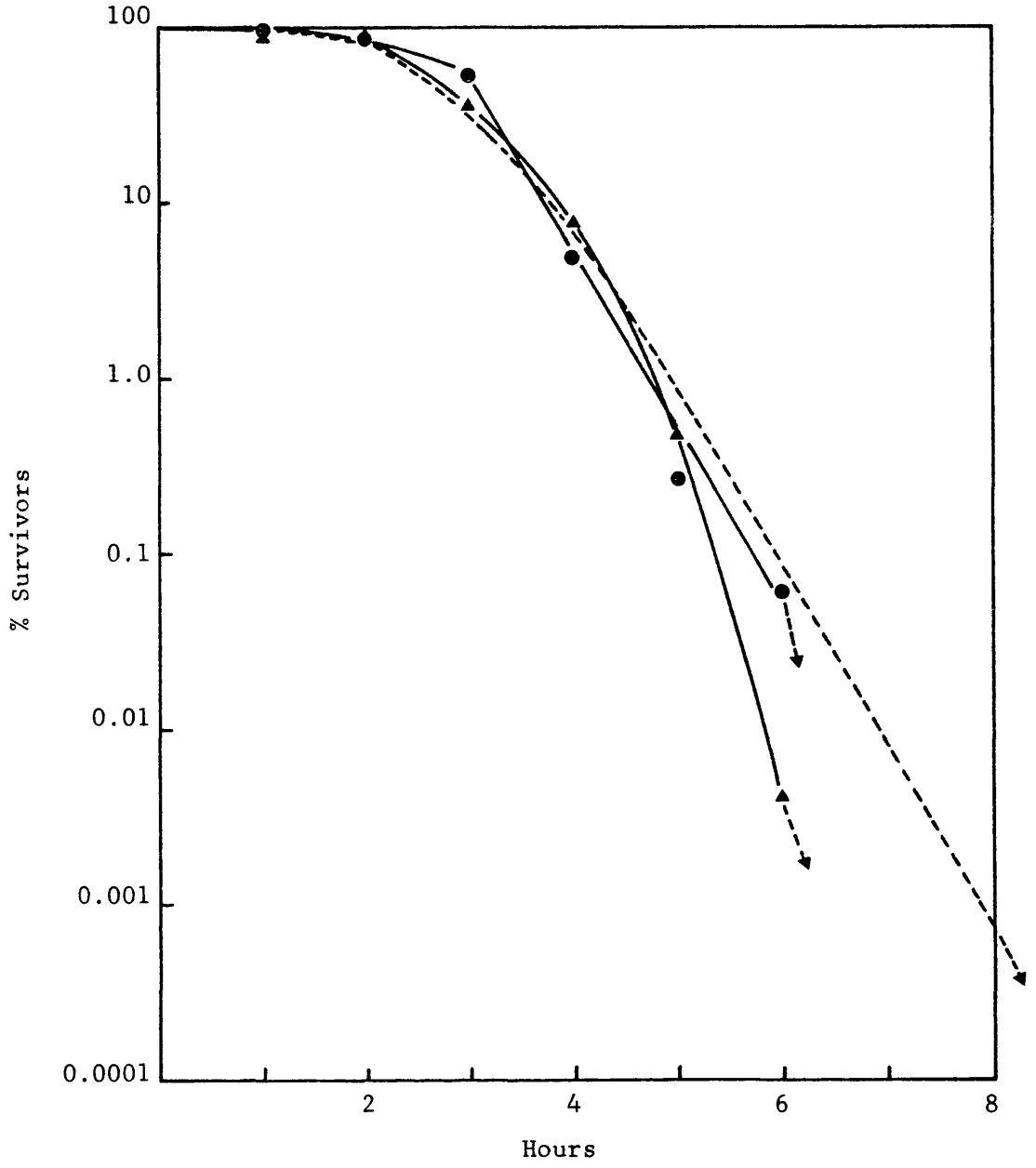


Fig.28 The recovery of E. coli on Columbia Agar Base (●), Nutrient Agar (▲) and Tryptone Soya Agar (---), after treatment with 0.008% w/v thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0, at 25°C.

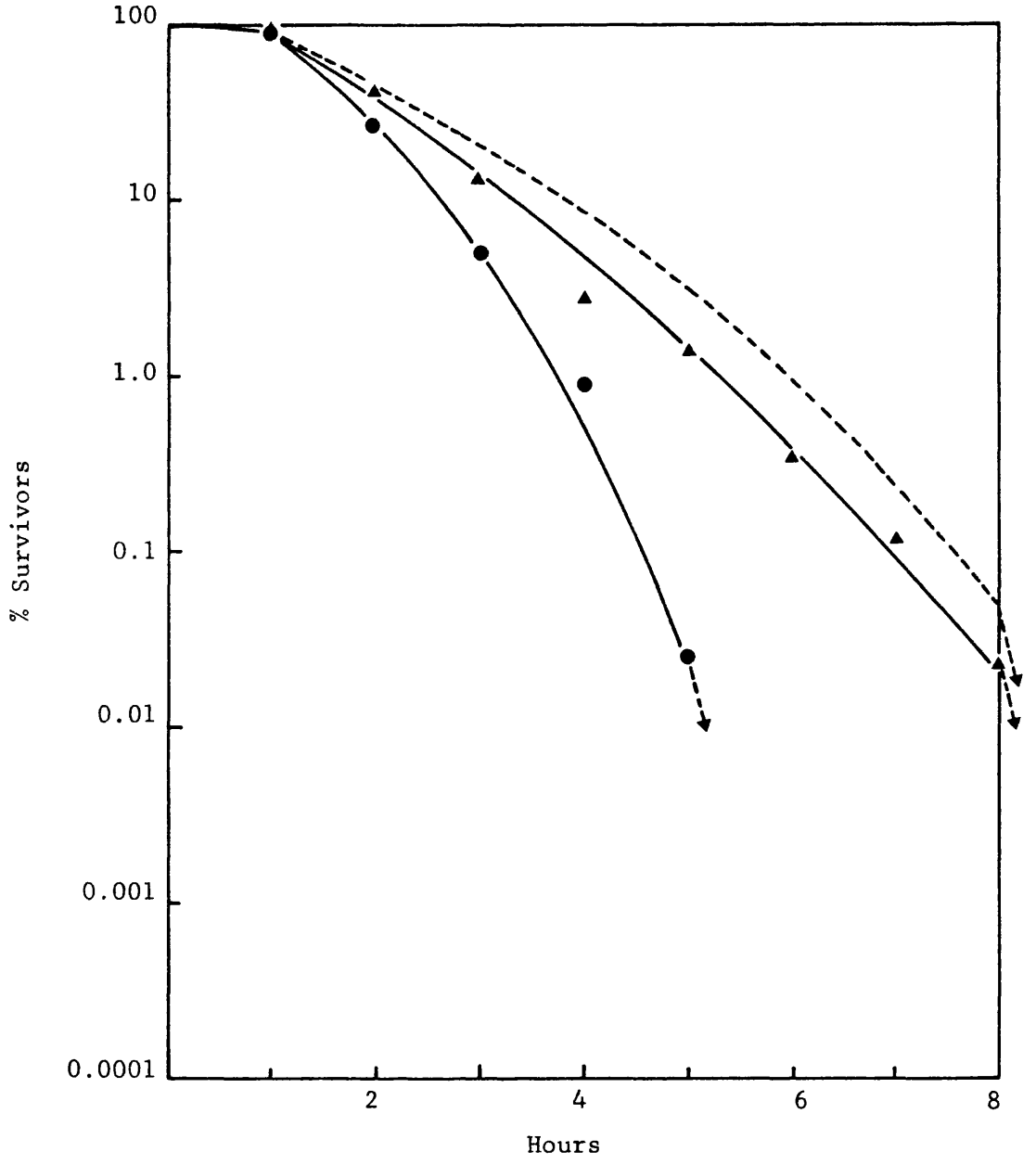


Fig.29 The recovery of C. albicans on Columbia Agar Base (●), Malt Extract Agar (▲) and Tryptone Soya Agar (---), after treatment with 0.008% w/v thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0, at 25°C.

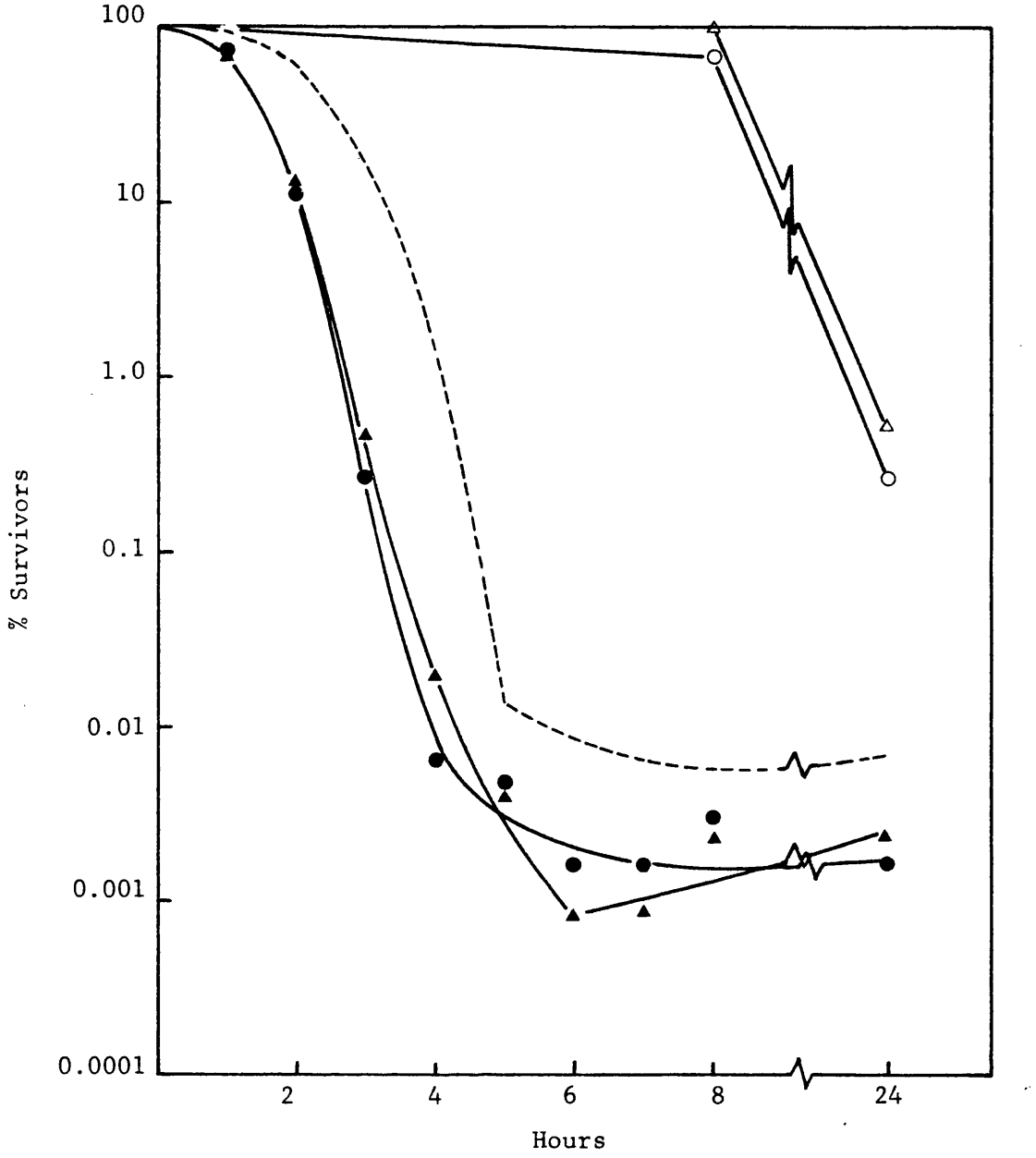


Fig.30 The recovery of B. subtilis on Columbia Agar Base (●), Nutrient Agar (▲) and Tryptone Soya Agar (---), after treatment with 0.008% w/v thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0, at 25°C.

Survival in buffer: recovery on CAB (○) and recovery on NA (△)

TABLE 24a

Colonial morphology of Staph. aureus where survivors had had 8h contact with thiomersal.

Plating Medium	Incubation Conditions	Description
CAB	24h at 37°C	Colonies: 2mm, glossy, entire-edged, flat with central dome, deep cream colour. Medium: unaffected.
	48h at 37°C	Colonies: 3-4mm; rest as for 24h.
NA	24h at 37°C	Colonies: 1mm, glossy, entire-edged, flat, pale cream colour. Medium: unaffected.
	48h at 37°C	Colonies: 2-3mm; rest as for 24h.

TABLE 24b

Colonial morphology of Ps. aeruginosa where survivors had had 1h contact with thiomersal.

Plating Medium	Incubation Conditions	Description
CAB	24h at 37°C	Colonies: 2-3mm, glossy, crenated-edged, umbonate, translucent, pale yellow-green colour. Medium: appeared unpigmented.
	48h at 37°C	Colonies: 5-7mm, irregular-shaped, yellow-brown colour. Medium: appeared unpigmented.

TABLE 24b (cont'd)

Plating Medium	Incubation Conditions	Description
NA	24h at 37°C	Colonies: 2-3mm, glossy, crenated-edged, umbonate, translucent, pale lime green colour. Medium: appeared unpigmented.
	48h at 37°C	Colonies: 3-4mm, slightly irregular in shape. Medium: some light green pigment present.

TABLE 24c

Colonial morphology of E. coli where survivors had had 2h contact with thiomersal.

Plating Medium	Incubation Conditions	Description
CAB	48h at 37°C	Colonies: 3mm, round, flat, glossy, entire-edged, sand-coloured. Medium: unaffected.
NA	48h at 37°C	Colonies: pale straw-coloured; rest as for CAB.

TABLE 24d

Colonial morphology of C. albicans where survivors had had ++2h contact with thiomersal.

Plating Medium	Incubation Conditions	Description
CAB	5 days at 37°C	Colonies: 2-3mm, round, entire-edged, glossy, domed, creamy-coloured. Medium: unaffected.
MEA	5 days at 37°C	Colonies: 4-5mm, round, entire-edged, opaque, pronounced dome, creamy-coloured. Medium: unaffected.

++An interesting phenomenon was observed with yeasts that had had 3h contact with thiomersal. On some of the colonies on the MEA plates, a change in the colonial morphology occurred. Instead of a central dome, a 'crinkled' area appeared on a raised central plateau. About half the colonies showed this change at 3h, but all survivors had this appearance by the end of the experiment. No such change was apparent with colonies that had grown from organisms in buffer only over the duration of the experiment.

TABLE 24e

Colonial morphology of B. subtilis where survivors had had 2h contact with thiomersal.

Plating Medium	Incubation Conditions	Description
CAB	24h at 37°C	Colonies: 4-6mm, crenated-edged, flat, deep orange centres with pale orange 'frills'. Medium: unaffected.
	48h at 37°C	Colonies: 15mm, very crenated edges; rest as for 24h.
NA	24h at 37°C	Colonies: 2-3mm, crenated-edged, flat, very pale creamy-brown. Medium: unaffected.
	48h at 37°C	Colonies: 6-7mm, very pale creamy-brown, with deeper brown centres; rest as for 24h.

TABLE 25

$t_{0.1}$ values recorded from the effect of plating media on the recovery of organisms treated with 0.008% w/v thiomersal.

Plating Media	$t_{0.1}$ values (hours)				
	Staph. aureus	Ps. aeruginosa	E. coli	C. albicans	B. subtilis
CAB	64.0	5.20	5.65	4.6	3.30
NA	65.0	4.75	5.30	N.D.	3.40
MEA	N.D.	N.D.	N.D.	7.0	N.D.
TSA	66.5	5.55	5.95	7.55	4.47

N.D. Not Done.

3) Effect of Composition of Thiomersal Test Solution

To examine the effect of changes in the composition of the experimental thiomersal solutions on the antimicrobial activity of thiomersal, boiled and cooled, sterilised distilled water (pH 6.5) and the appropriate growth medium for the test organism concerned, were compared with isotonic Sörensen's phosphate buffer (pH 7.0).

Figures 31-35 illustrate the results obtained with these experiments and the $t_{0.1}$ values recorded are presented in Table 26. The survival of the test organisms in distilled water and growth media over the duration of the experiment are depicted in Figure 36.

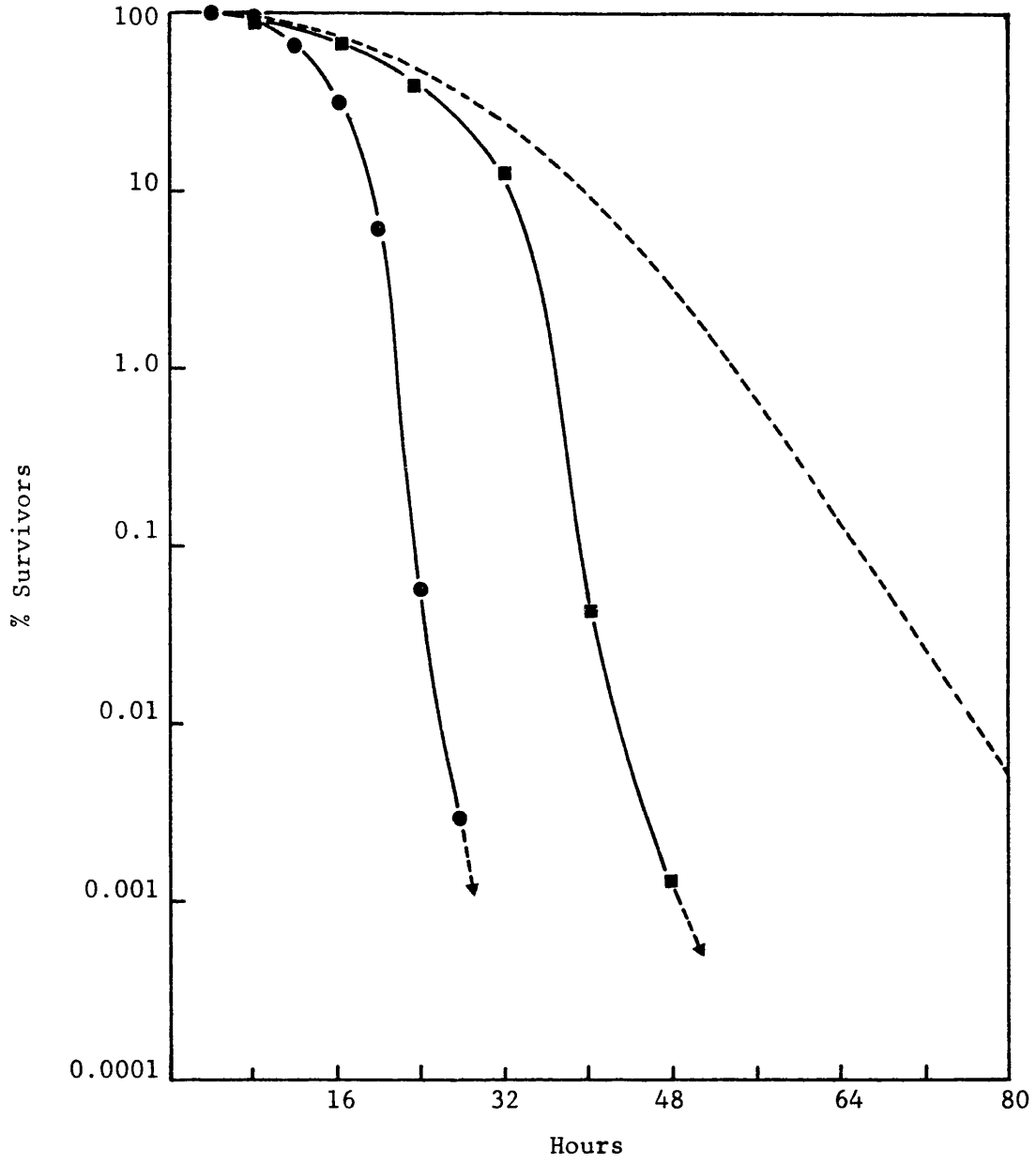


Fig.31 Effect of 0.008% w/v thiomersal in distilled water, pH 6.5 (●), chemically-defined medium, pH 7.0 (■) and isotonic Sörensen's phosphate buffer, pH 7.0 (---) at 25°C, on Staph. aureus.

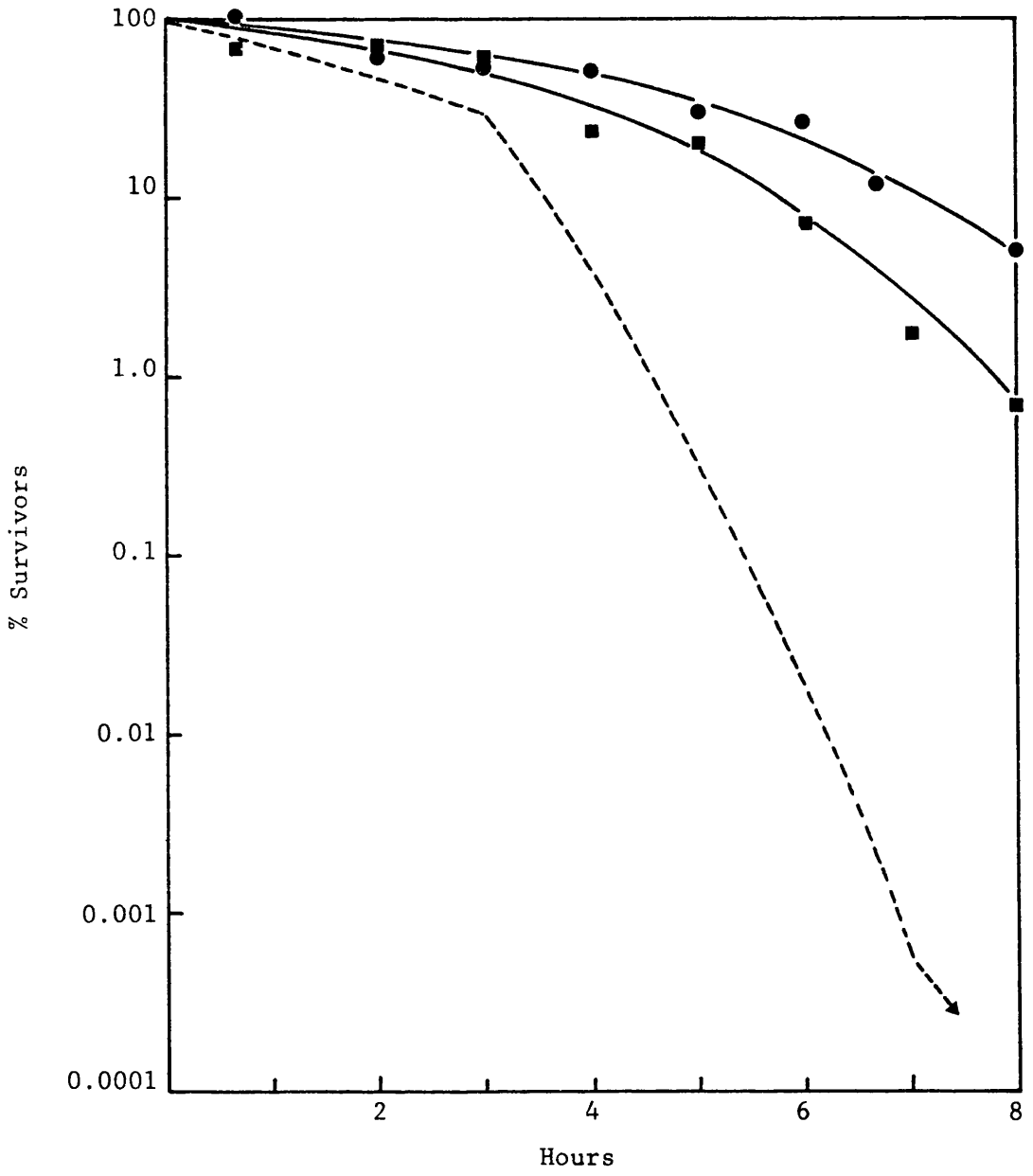


Fig.32 Effect of 0.008% w/v thiomersal in distilled water, pH 6.5 (●), chemically-defined medium, pH 7.0 (■) and isotonic Sörensen's phosphate buffer, pH 7.0 (---) at 25°C, on Ps. aeruginosa.

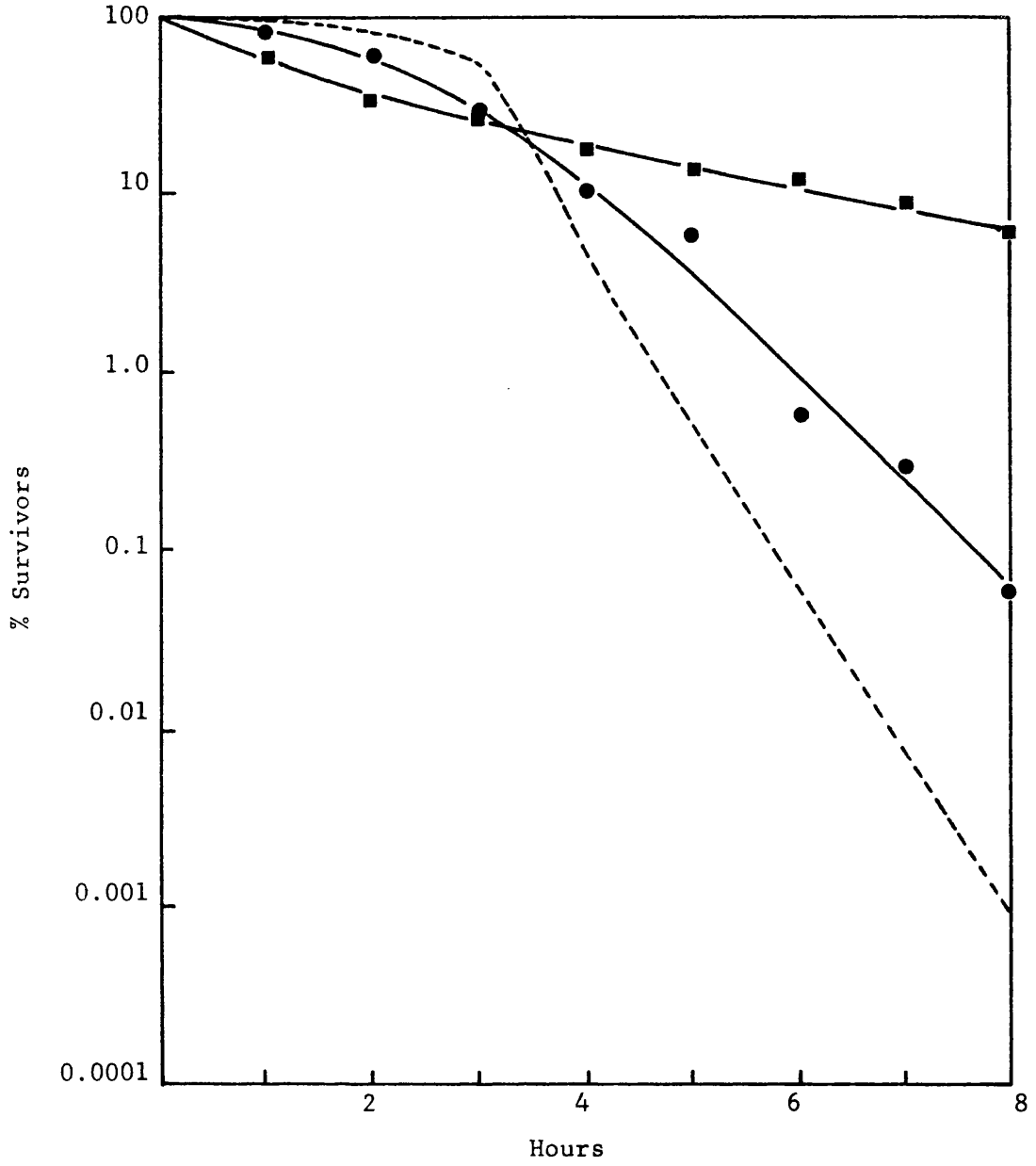


Fig.33 Effect of 0.008% w/v thiomersal in distilled water, pH 6.5 (●), chemically-defined medium, pH 7.0 (■) and isotonic Sörensen's phosphate buffer, pH 7.0 (---) at 25°C, on E. coli.

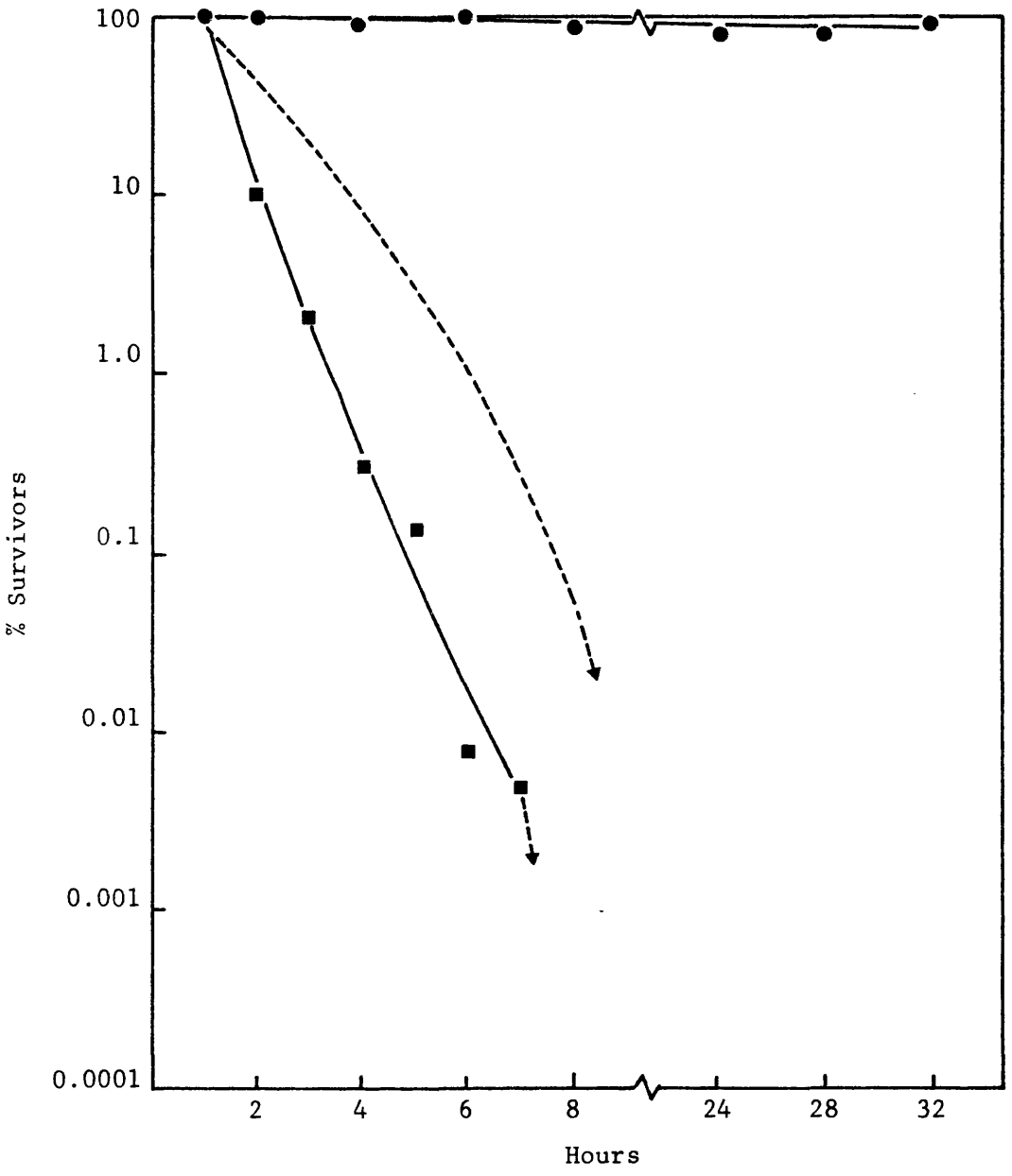


Fig.34 Effect of 0.008% w/v thiomersal in distilled water, pH 6.5 (●), chemically-defined medium, pH 7.0 (■) and isotonic Sörensen's phosphate buffer, pH 7.0 (---) at 25°C, on C. albicans.

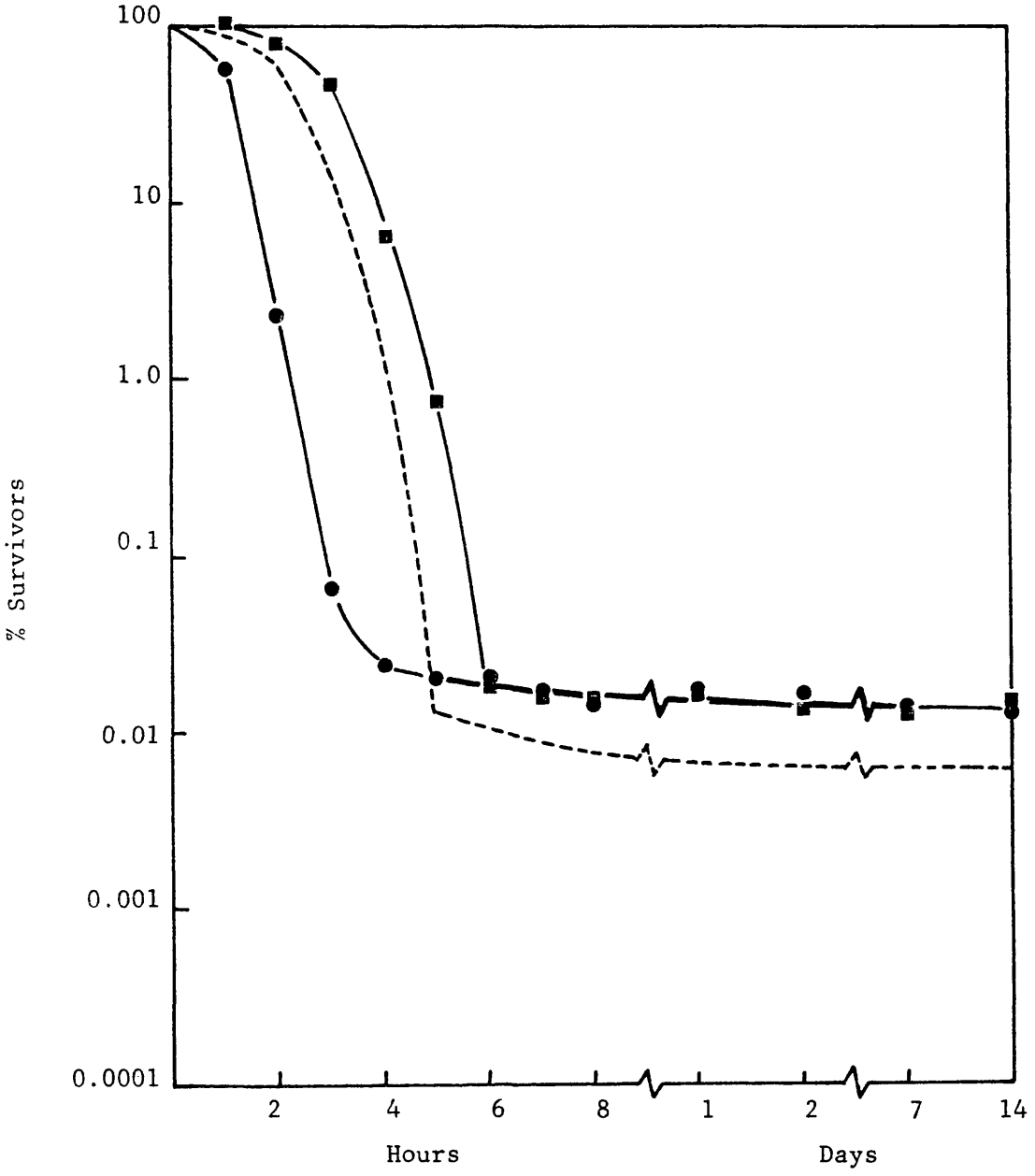


Fig.35 Effect of 0.008% w/v thiomersal in distilled water, pH 6.5 (●), chemically-defined medium, pH 7.0 (■) and isotonic Sörensen's phosphate buffer, pH 7.0 (---) at 25°C, on B. subtilis.

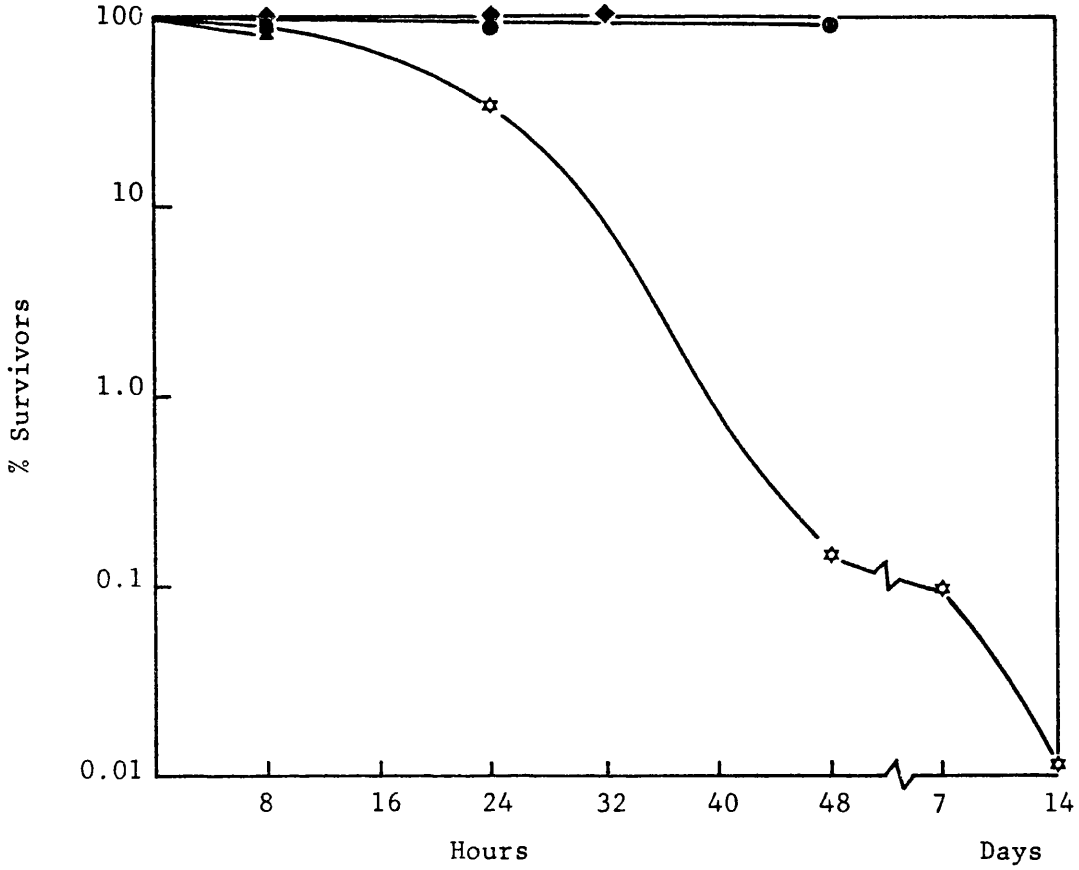


Fig.36a Survival in distilled water.

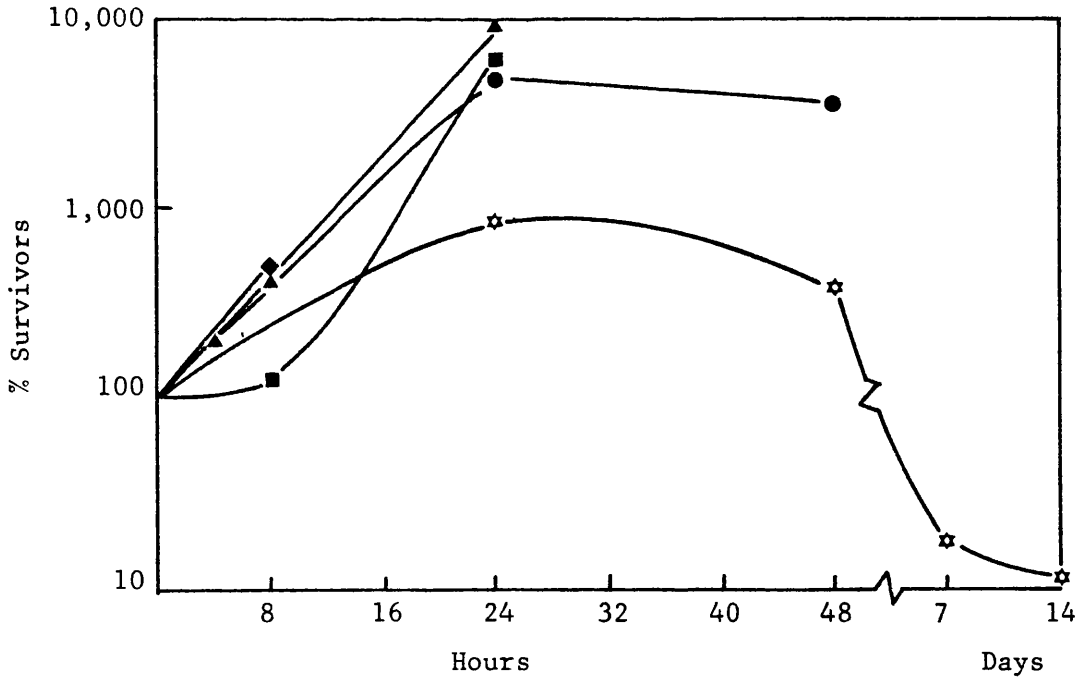


Fig.36b Survival in growth media.

Fig.36 Survival of test organisms in distilled water and appropriate growth media at 25°C. Staph. aureus (●), Ps. aeruginosa (▲), E. coli (■), C. albicans (◆), B. subtilis (✡)

TABLE 26

‡‡$t_{0.1}$ values recorded from the effect of composition of thiomersal test solutions on its antimicrobial activity .

Composition of Thiomersal Solution	$t_{0.1}$ values (hours)				
	Staph. aureus	‡‡Ps. aeruginosa	‡‡E. coli	C. albicans	B. subtilis
Sterile distilled water (pH 6.5)	24.0	7.25	4.1	>32	2.8
Isotonic Sörensen's phosphate buffer (pH 7.0)	66.5	3.65	3.7	7.6	4.4
Chemically defined growth medium (pH 7.0)	39.5	5.60	6.4	4.8	5.4

‡‡t_{10} values read for Ps. aeruginosa and E. coli.

4) Effect of Tonicity

To determine the effect of tonicity on the antimicrobial activity of thiomersal, two further buffered solutions were prepared to represent hypertonicity (1.43 sodium chloride equivalent) and hypotonicity (no sodium chloride), using Sörensen's phosphate buffer, pH 7.0.

Figures 37-41 depict the results of these investigations and the $t_{0.1}$ values obtained are recorded in Table 27.

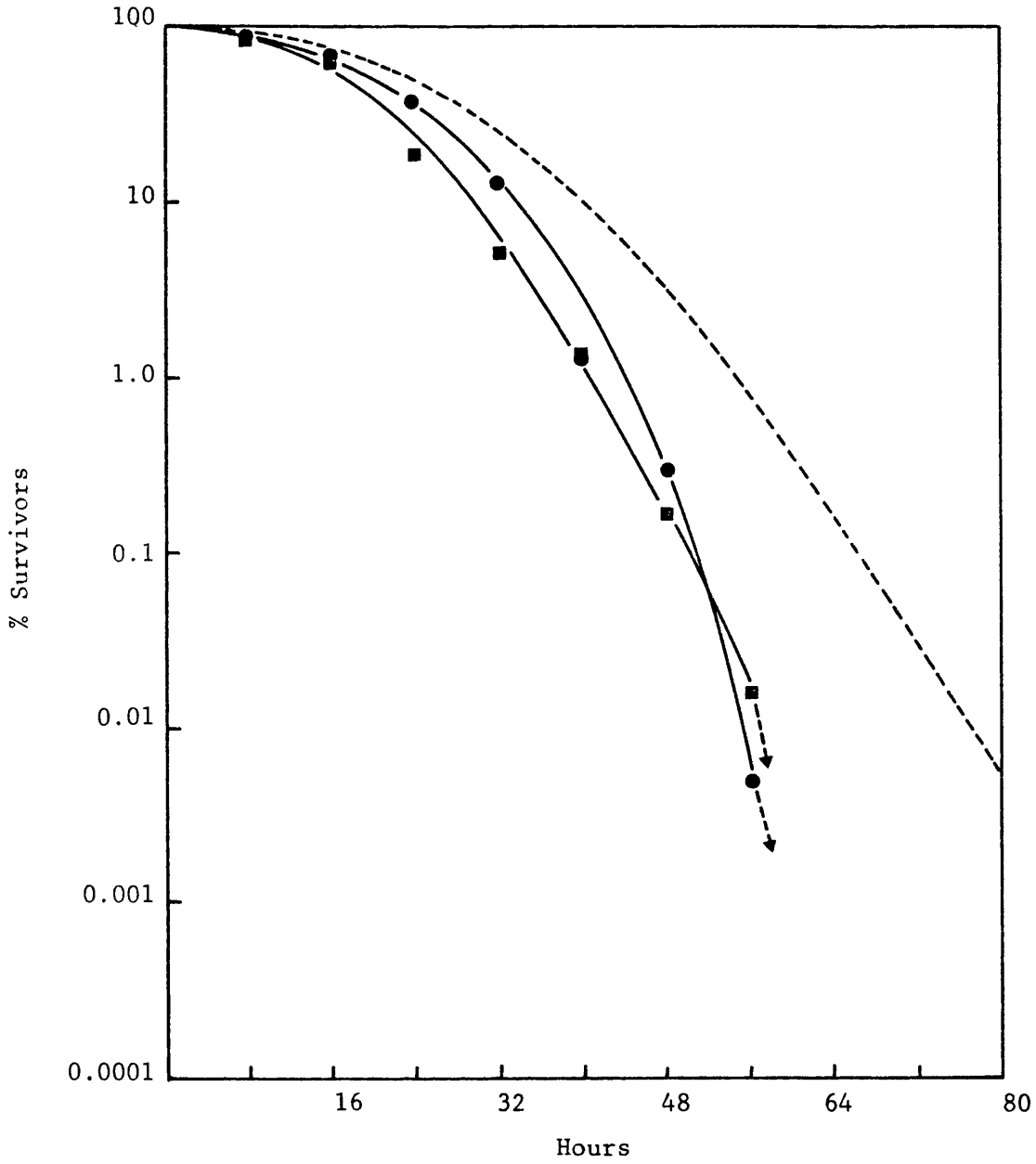


Fig.37 Effect of 0.008% w/v thiomersal in hypotonic (●), isotonic (---) and hypertonic (■) Sörensen's phosphate buffer, pH 7.0, at 25°C on Staph. aureus.

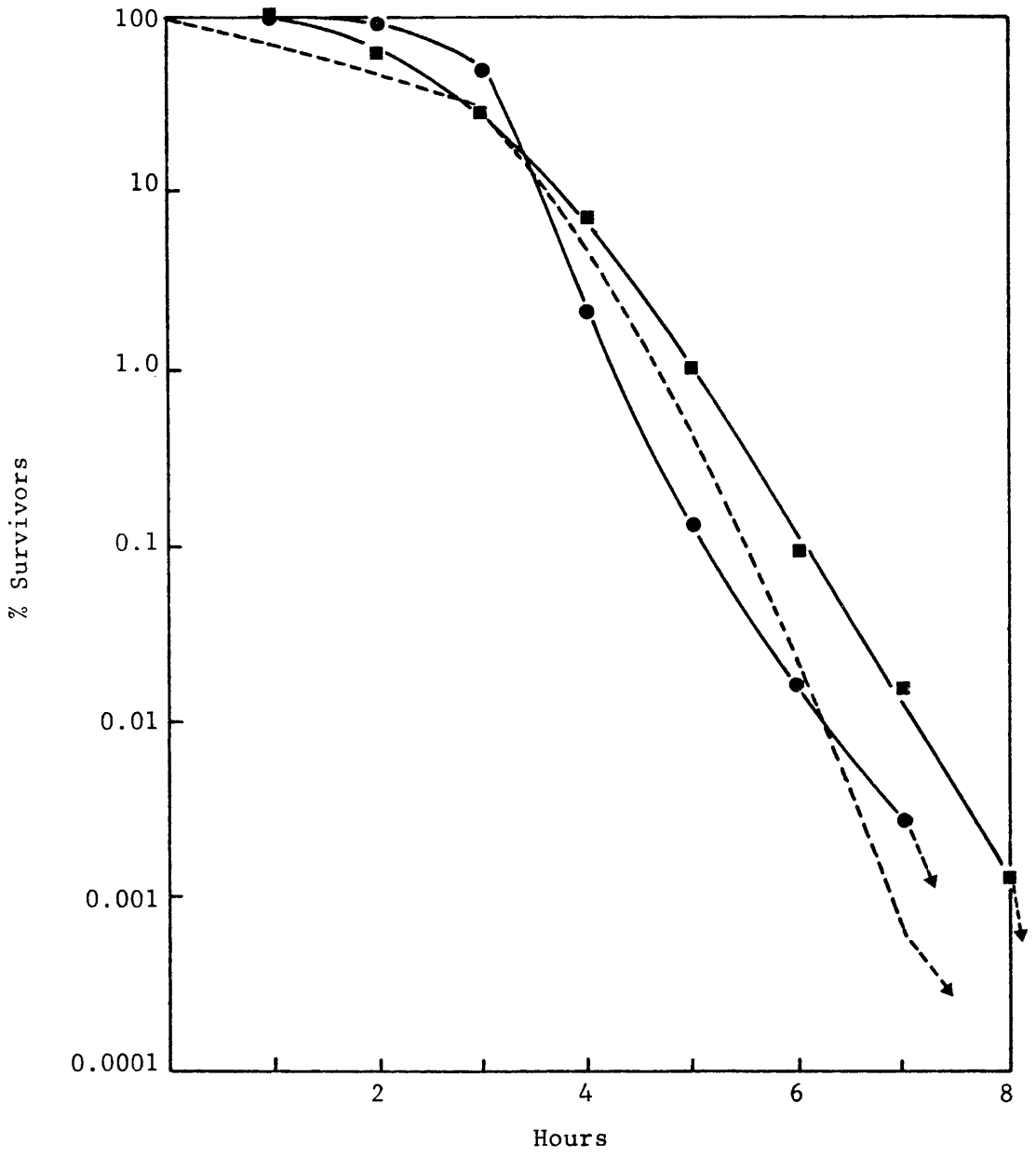


Fig.38 Effect of 0.008% w/v thiomersal in hypotonic (●), isotonic (---) and hypertonic (■) Sörensen's phosphate buffer, pH 7.0, at 25°C on Ps. aeruginosa.

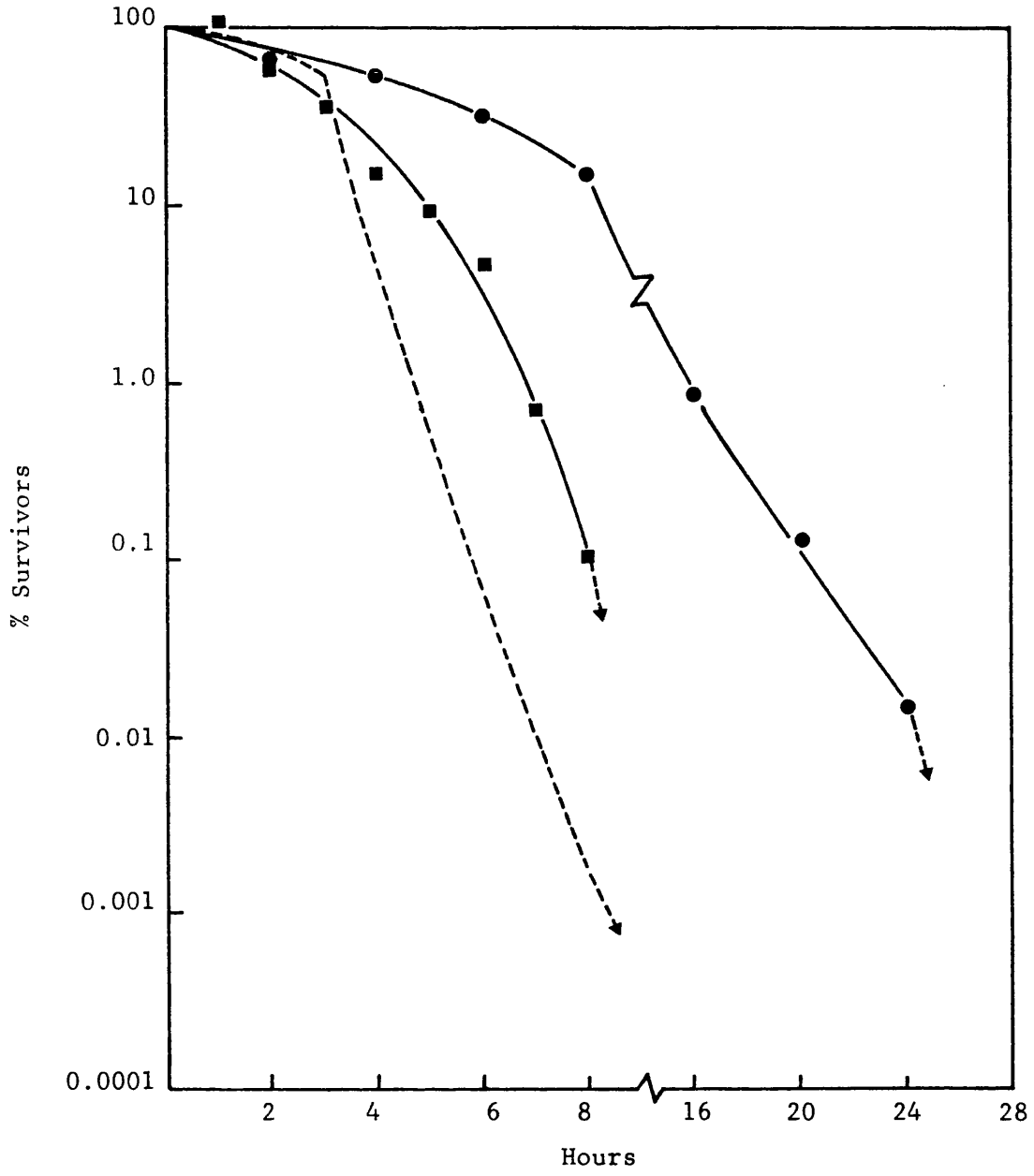


Fig.39 Effect of 0.008% w/v thiomersal in hypotonic (●), isotonic (---) and hypertonic (■) Sörensen's phosphate buffer, pH 7.0, at 25°C on E. coli.

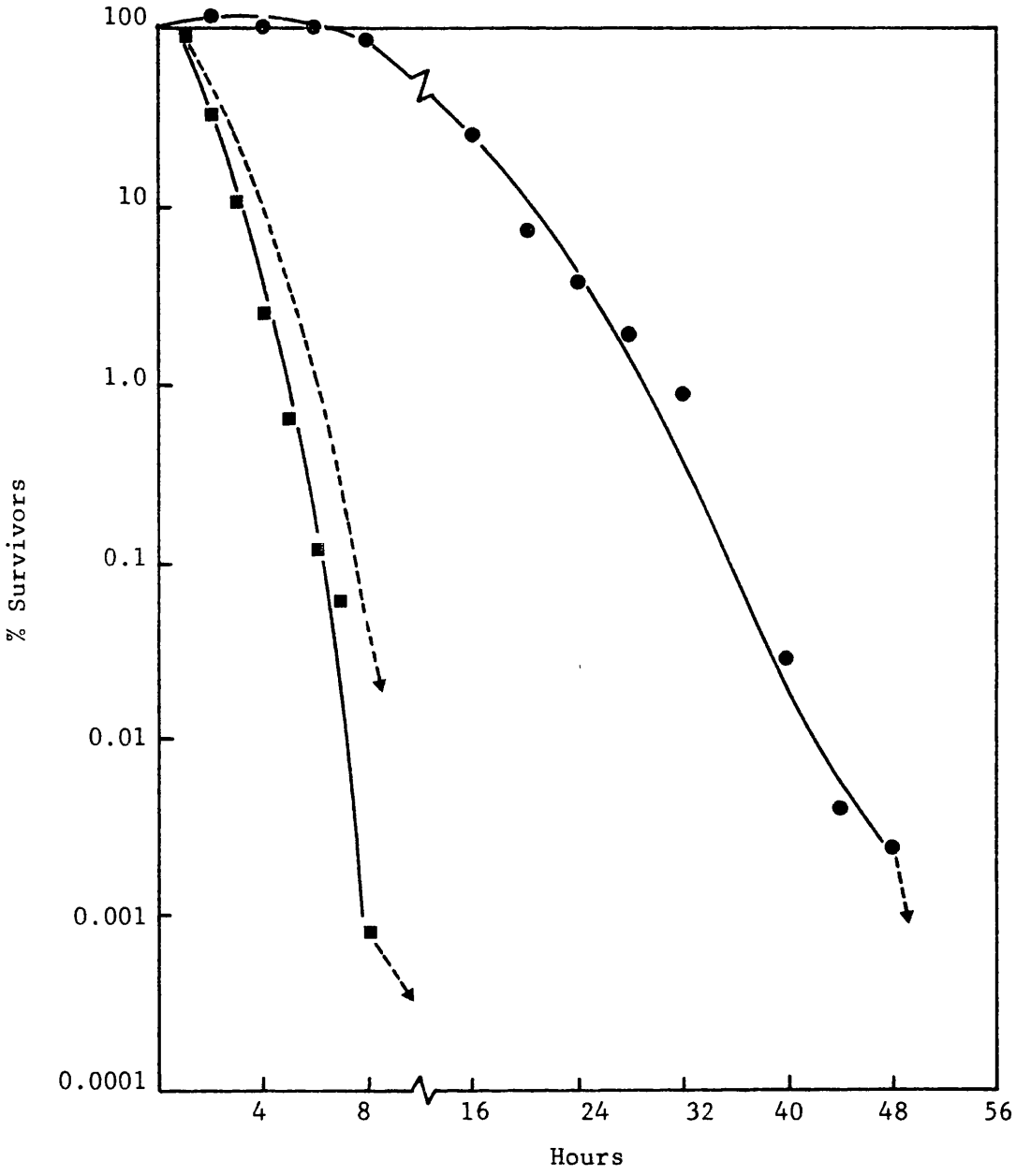


Fig.40 Effect of 0.008% w/v thiomersal in hypotonic (●), isotonic (←--) and hypertonic (■) Sörensen's phosphate buffer, pH 7.0, at 25°C on C. albicans.

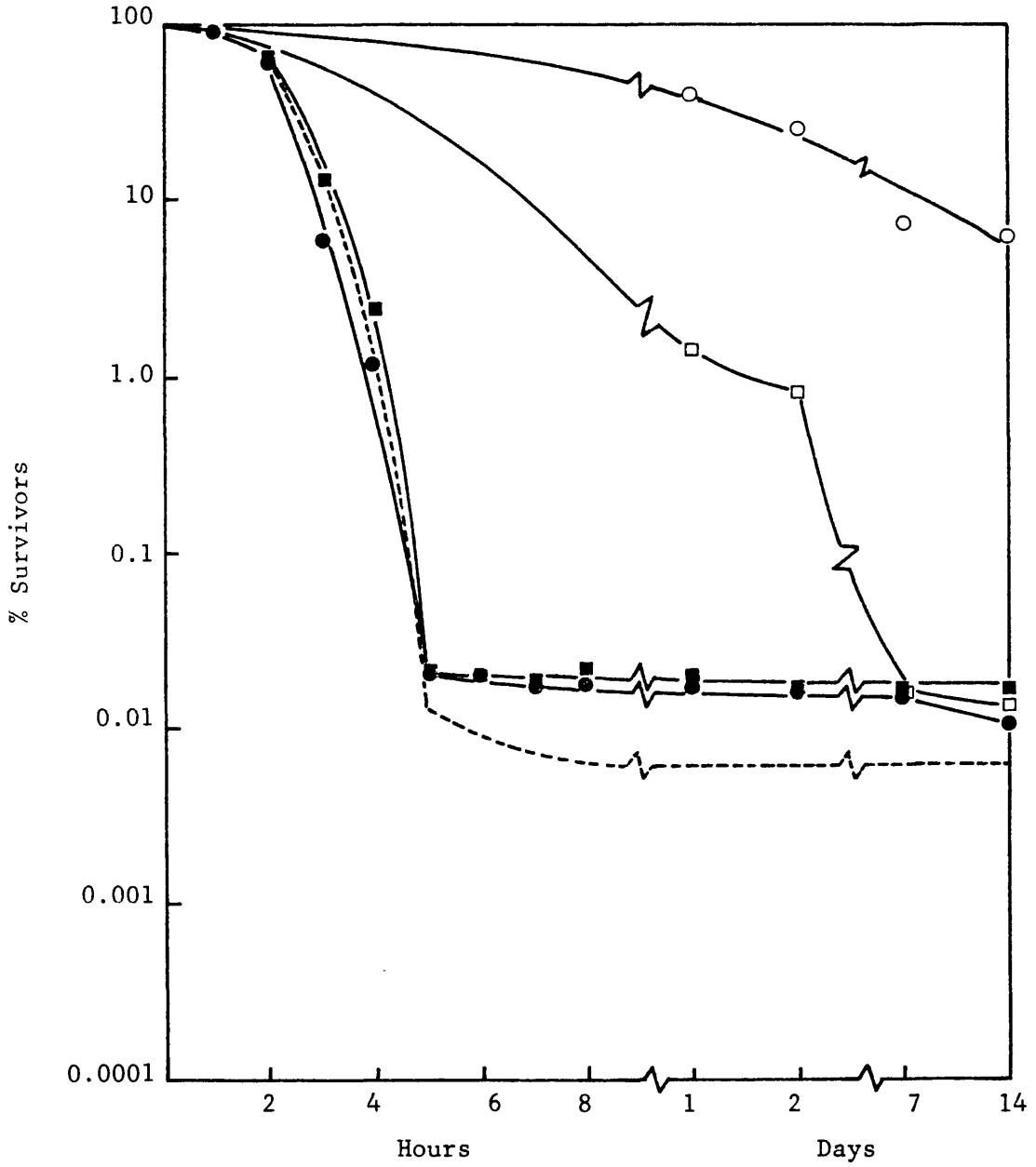


Fig.41 Effect of 0.008% w/v thiomersal in hypotonic (●), isotonic (---) and hypertonic (■) Sorensen's phosphate buffer, pH 7.0, at 25°C on B. subtilis.

Survival in buffer: hypotonic (○) and hypertonic (□)

TABLE 27

$t_{0.1}$ values recorded from the effect of tonicity on the antimicrobial activity of thiomersal.

Tonicity Status	$t_{0.1}$ values (hours)				
	Staph. aureus	Ps. aeruginosa	E. coli	C. albicans	B. subtilis
Hypotonic	50.0	5.20	20.2	36.0	4.4
Isotonic	66.5	5.55	5.9	7.5	4.4
Hypertonic	49.0	6.10	8.0	6.3	4.8

5) Effect of pH

Freshly-prepared Sörensen's phosphate buffers were made to cover the desired range of pH, namely 4.5, 5.0, 6.0 and 8.0. All solutions were made isotonic with sodium chloride before being used to prepare the thiomersal test solutions.

Figures 42-46 illustrate the effect of pH on the antimicrobial activity of thiomersal. pH measurements on test solutions carried out at the end of each experiment showed that there was very little change in pH over an experiment. The $t_{0.1}$ values obtained are listed in Table 28.

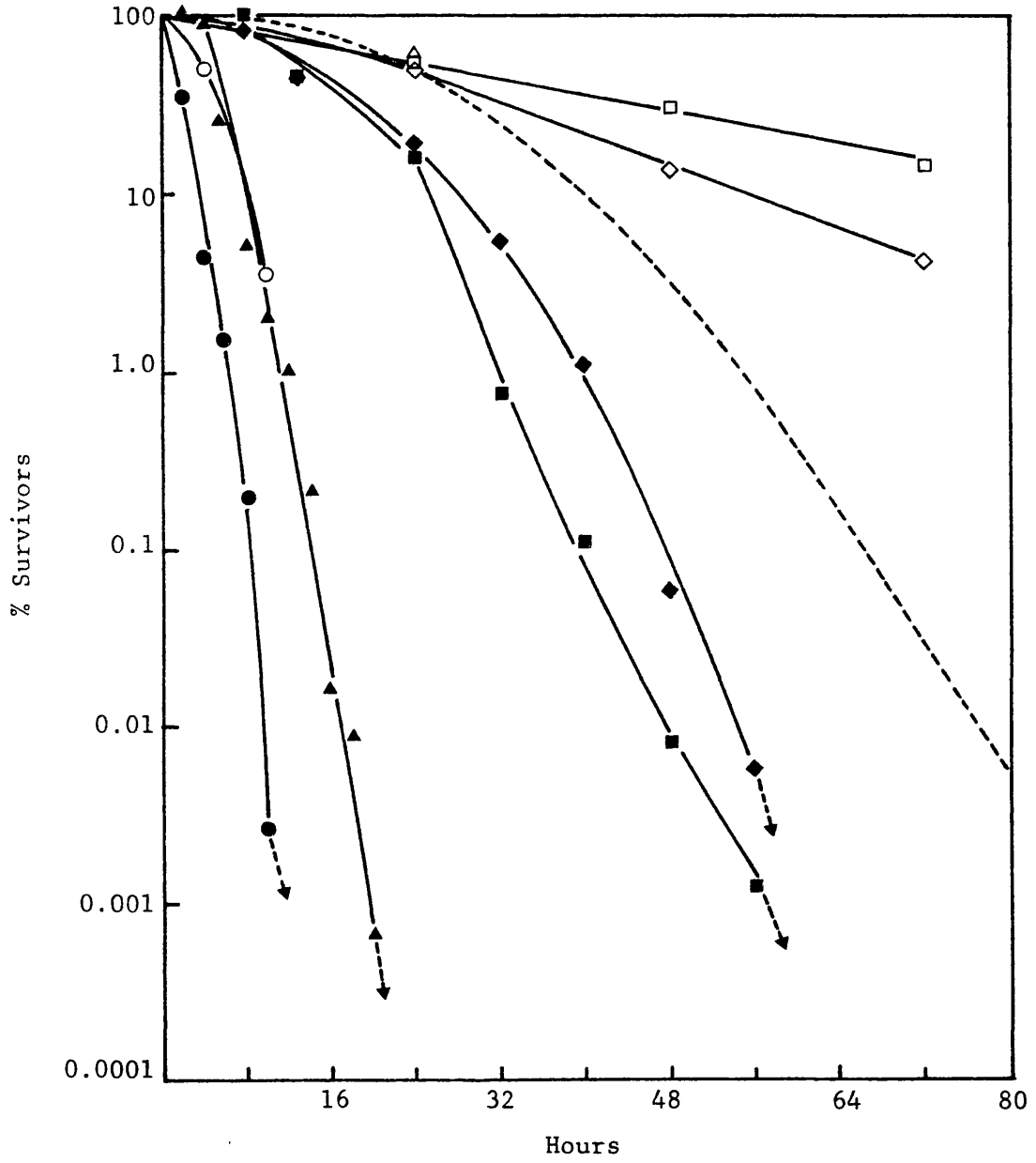


Fig.42 Effect of 0.008% w/v thiomersal in isotonic Sörensen's phosphate buffer, pH 4.5 (●), pH 5.0 (▲), pH 6.0 (■), pH 7.0 (---) and pH 8.0 (◆), on Staph. aureus at 25°C.

Survival in buffer: pH 4.5 (○), pH 5.0 (△), pH 6.0 (□) and pH 8.0 (◇).

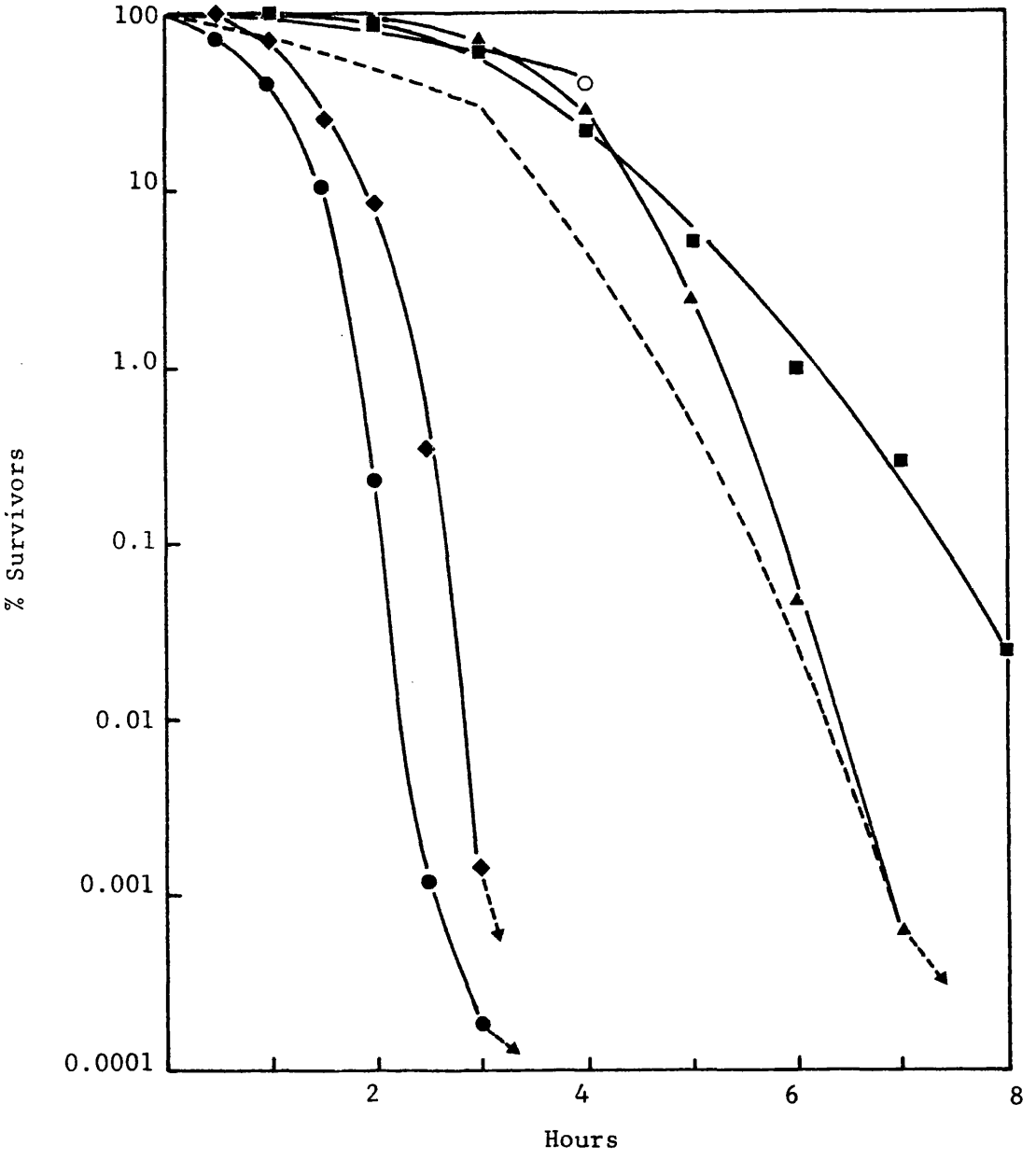


Fig.43 Effect of 0.008% w/v thiomersal in isotonic Sörensen's phosphate buffer, pH 4.5 (●), pH 5.0 (▲), pH 6.0 (■), pH 7.0 (---) and pH 8.0 (◆), on Ps. aeruginosa at 25°C.

Survival in buffer: pH 4.5 (○)

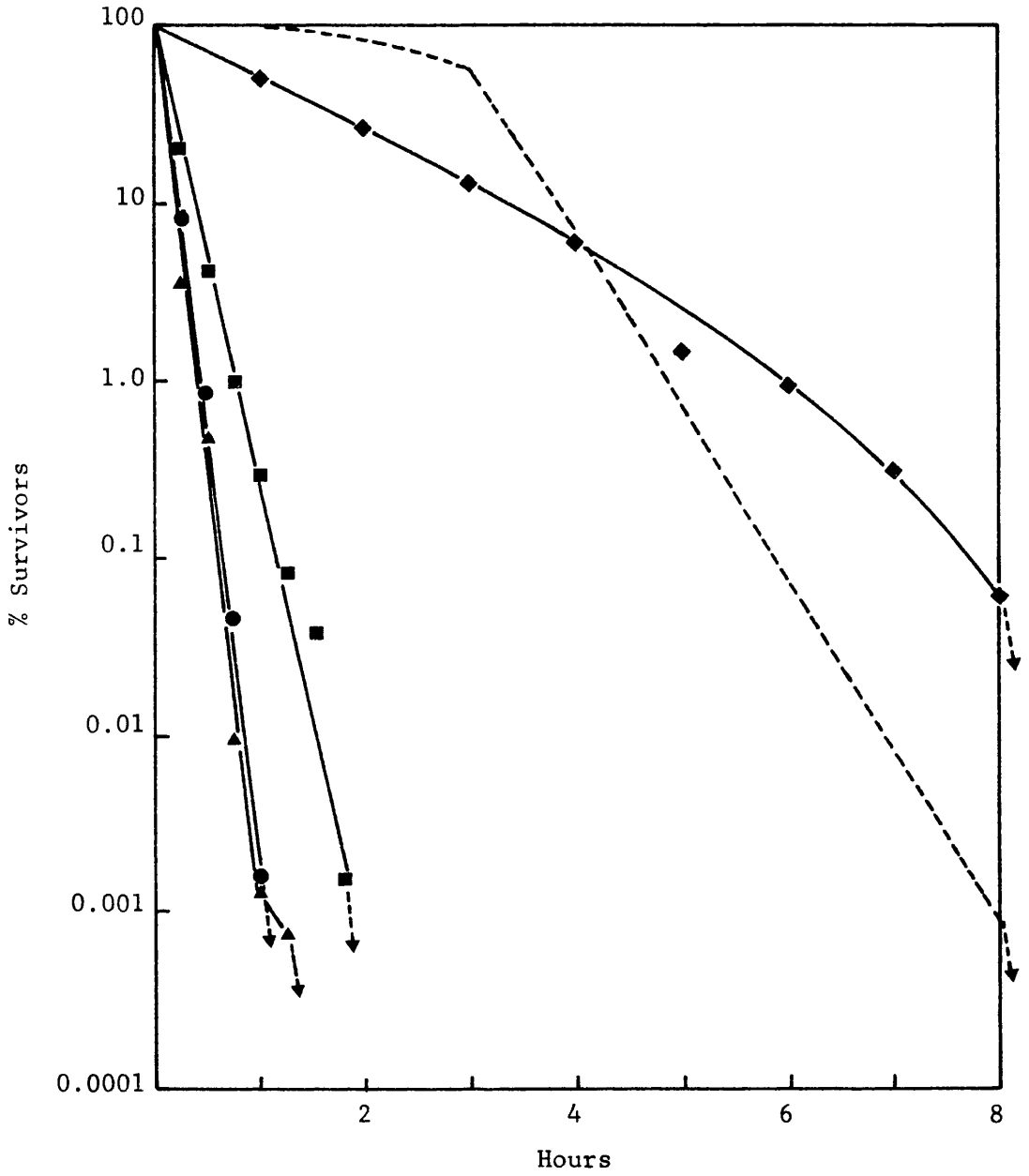


Fig.44 Effect of 0.008% w/v thiomersal in isotonic Sörensen's phosphate buffer, pH 4.5 (●), pH 5.0 (▲), pH 6.0 (■), pH 7.0 (---) and pH 8.0 (◆) on E. coli at 25°C.

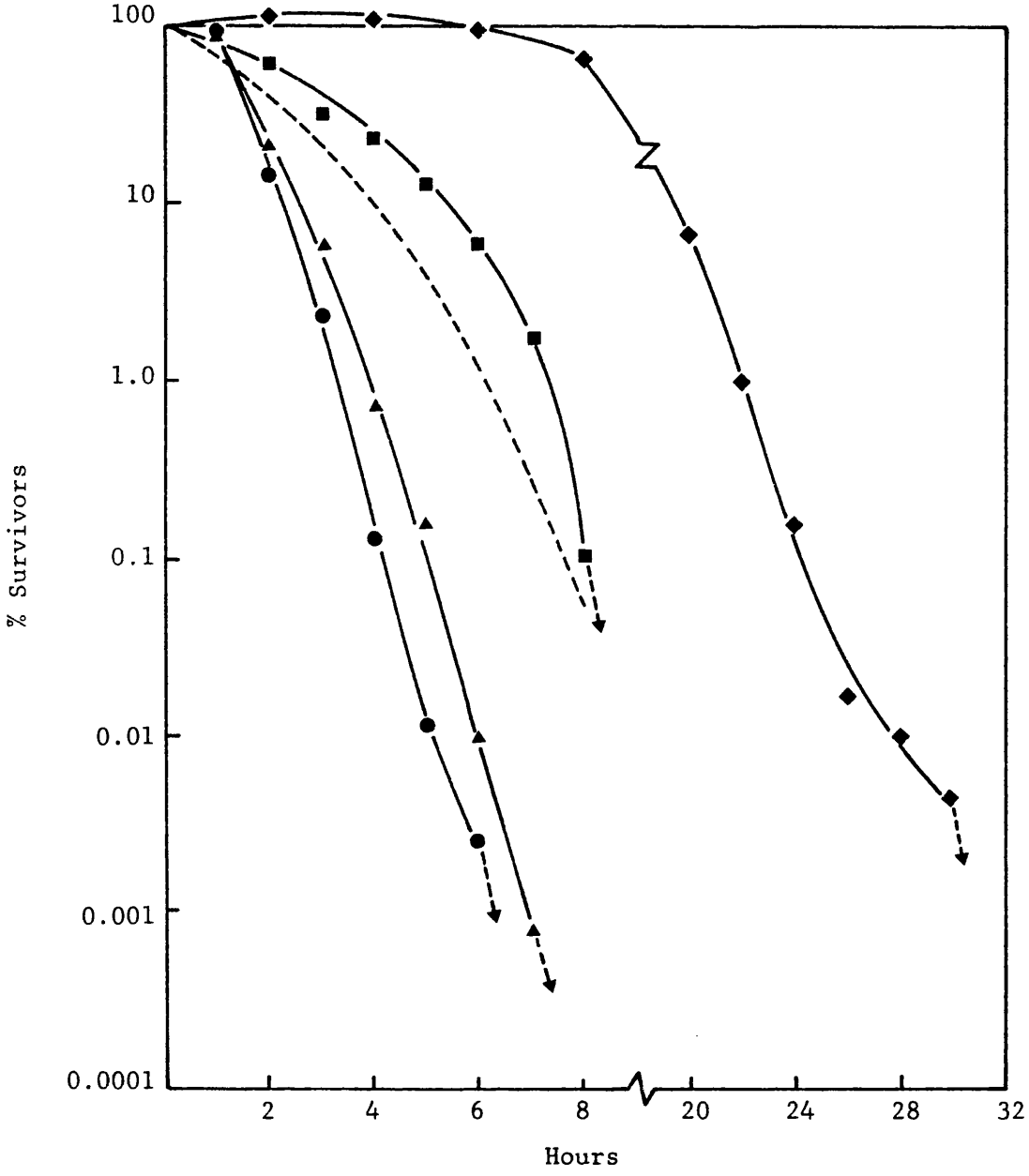


Fig.45 Effect of 0.008% w/v thiomersal in isotonic Sörensen's phosphate buffer, pH 4.5 (●), pH 5.0 (▲), pH 6.0 (■), pH 7.0 (---) and pH 8.0 (◆) on C. albicans at 25°C.

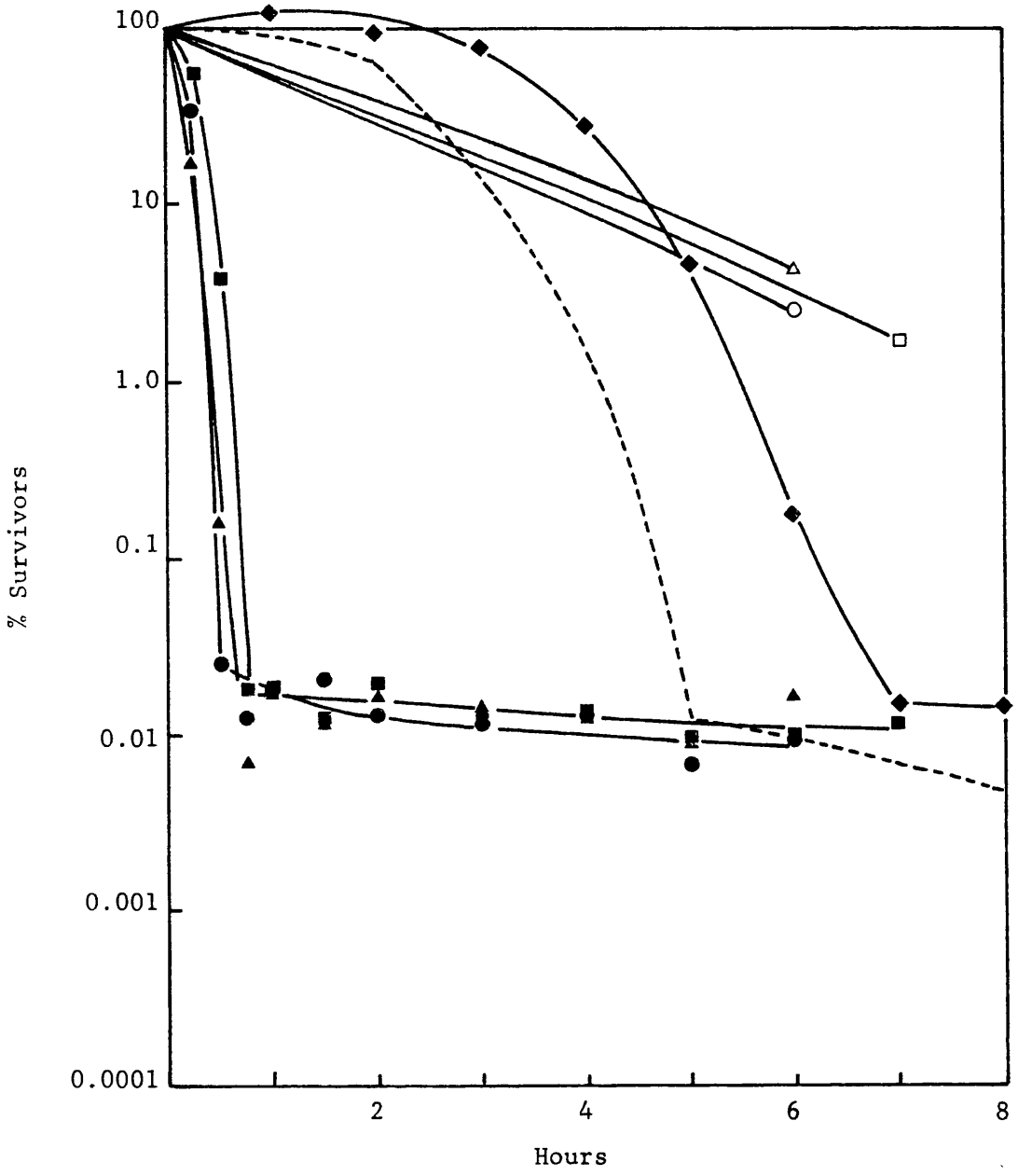


Fig.46 Effect of 0.008% w/v thiomersal in isotonic Sörensen's phosphate buffer, pH 4.5 (●), pH 5.0 (▲), pH 6.0 (■), pH 7.0 (---) and pH 8.0 (◆) on B. subtilis at 25°C.

Survival in buffer: pH 4.5 (○), pH 5.0 (△) and pH 6.0 (□)

TABLE 28

$t_{0.1}$ values recorded from the effect of pH on the antimicrobial activity of thiomersal.

pH of Test Solution	$t_{0.1}$ values (hours)				
	Staph. aureus	Ps. aeruginosa	E. coli	C. albicans	B. subtilis
4.5	8.0	2.05	0.60	4.1	0.40
5.0	14.0	5.90	0.55	5.1	0.55
6.0	39.0	7.40	1.20	8.0	0.65
7.0	66.5	5.50	5.95	7.6	4.40
8.0	47.0	2.70	7.70	24.4	6.20

6) Effect of Temperature

Test solutions of thiomersal were prepared in isotonic Sörensen's phosphate buffer, pH 7.0, as normal, and allowed to equilibrate at the temperatures selected for testing, namely, 15°C, 20°C and 30°C. Test suspensions of the organisms, prepared as usual, were also allowed to equilibrate at these temperatures before being used in the challenge experiments, to eliminate the possibility of any initial temperature shock occurring.

Figures 47-51 illustrate the effect of temperature on the antimicrobial activity of thiomersal. Tables 29 and 30 present the $t_{0.1}$ values recorded and the temperature coefficients calculated using equation 5, respectively.

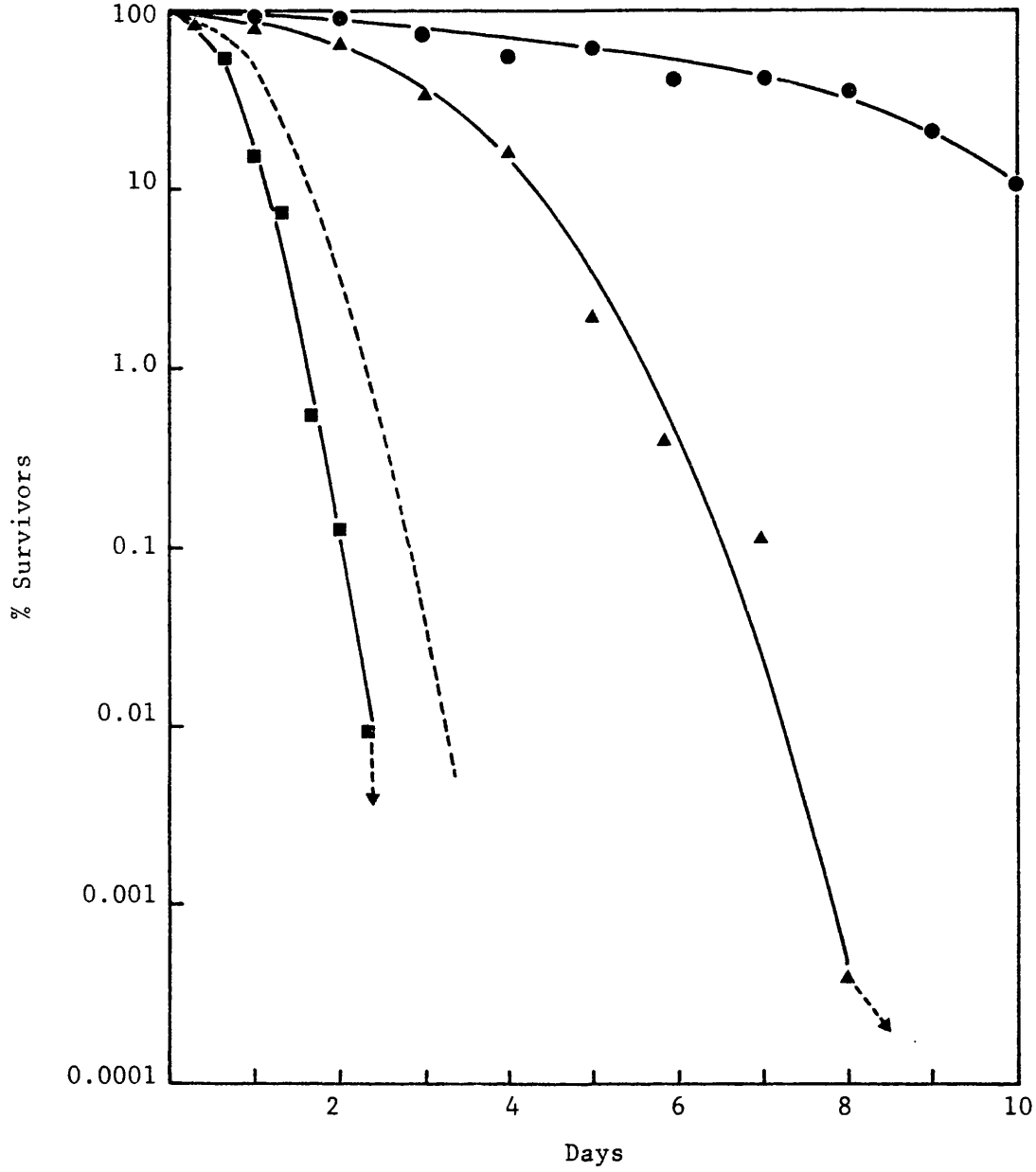


Fig.47 Effect of 0.008% w/v thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0, at 15°C (●), 20°C (▲), 25°C (---) and 30°C (■) on Staph. aureus.

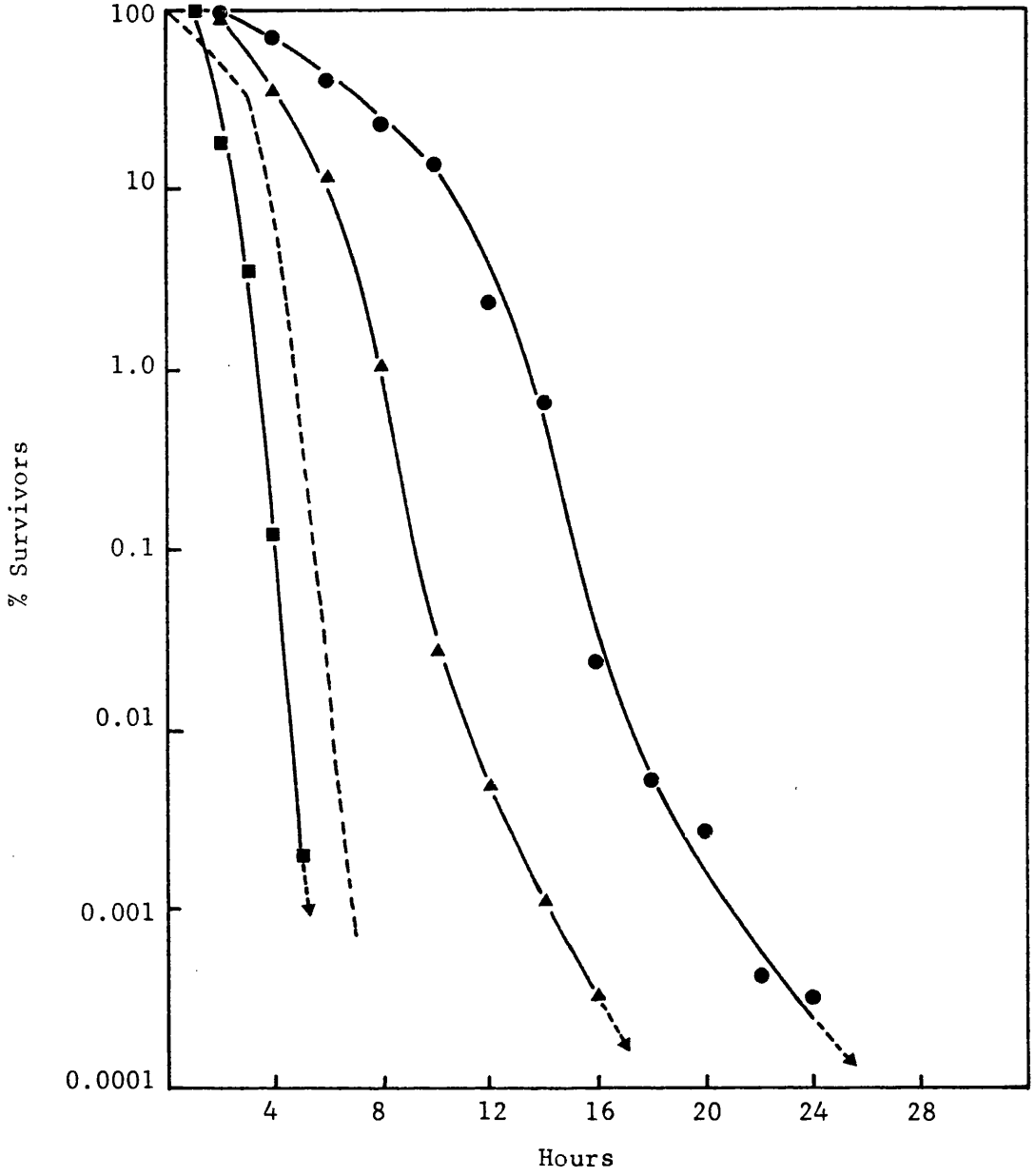


Fig.48 Effect of 0.008% w/v thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0, at 15°C (●), 20°C (▲), 25°C (---) and 30°C (■) on Ps. aeruginosa.

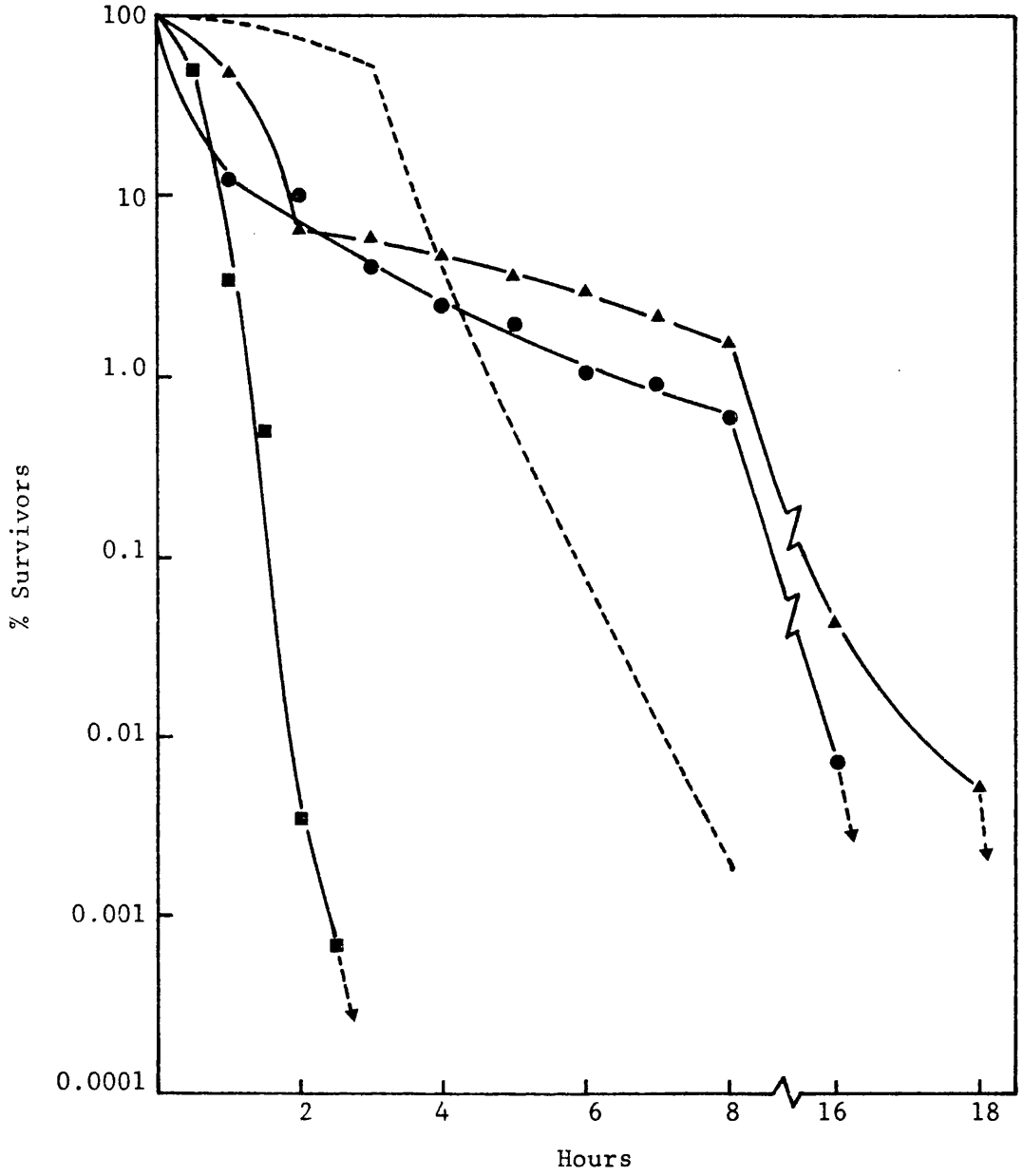


Fig.49 Effect of 0.008% w/v thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0, at 15°C (●), 20°C (▲), 25°C (---) and 30°C (■) on E. coli.

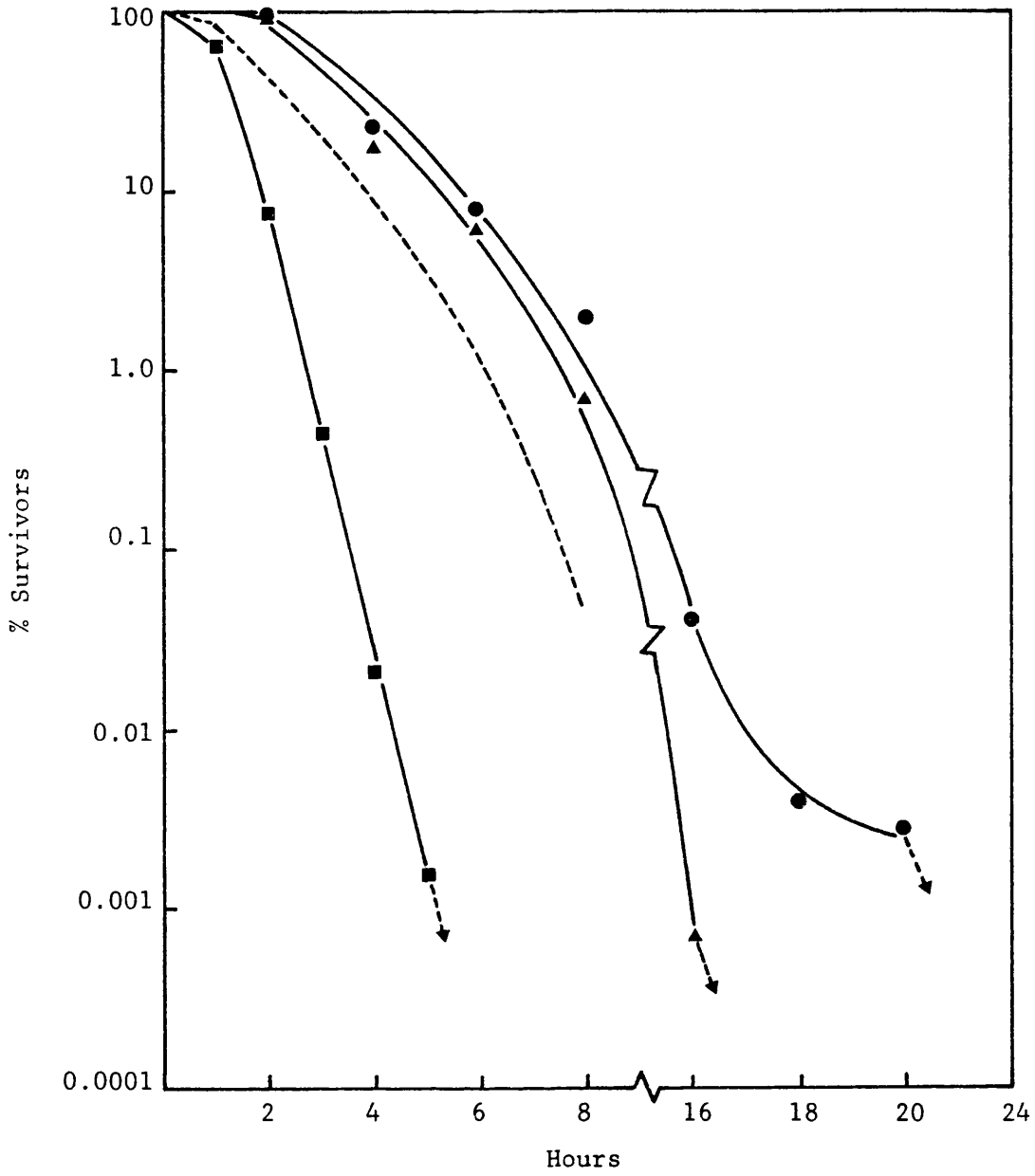


Fig.50 Effect of 0.008% w/v thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0, at 15°C (●), 20°C (▲), 25°C (---) and 30°C (■) on C. albicans.

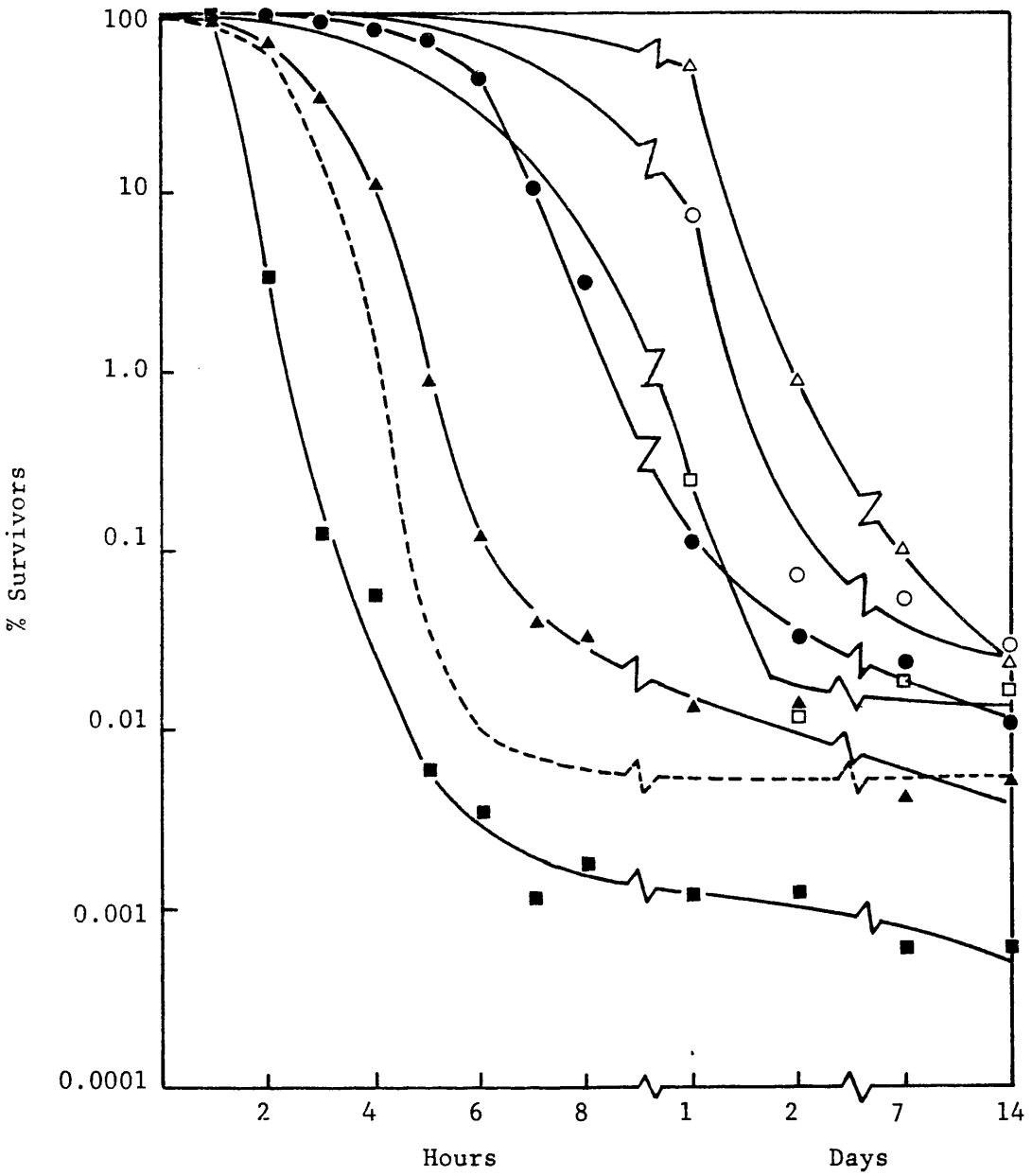


Fig.51 Effect of 0.008% w/v thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0, at 15°C (●), 20°C (▲), 25°C (---) and 30°C (■) on B. subtilis.

Survival in buffer: 15°C (○), 20°C (△) and 30°C (□)

TABLE 29

‡‡$t_{0.1}$ values recorded from the effect of temperature on the antimicrobial activity of thiomersal.

Temperature of Waterbath (°C)	$t_{0.1}$ values (hours) Staph. aureus	Ps. aeruginosa	‡‡E. coli	‡‡C. albicans	B. subtilis
15	> 264	15.2	6.4	8-16	26.4
20	156.8	9.2	8-16	7.6	6.2
25	65.6	5.5	4.7	6.0	4.4
30	48.8	4.0	1.2	2.7	3.3

‡‡$t_{1.0}$ values read for E. coli and C. albicans.

TABLE 30

Temperature coefficients (Q_{10} values) calculated for the test organisms.

Test Organism	Q_{10} values[++]	Average Q_{10} value
Staph. aureus	3.2	
Ps. aeruginosa	2.3	
E. coli	1.3	2.3
C. albicans	2.8	
B. subtilis	1.8	

[++] Calculated by substitution in equation 5, page 40, using temperatures of 20°C and 30°C (15°C and 25°C for E. coli).

7) Effect of Age of Test Culture

To determine the effect of this parameter on the antimicrobial activity of thiomersal, cells were harvested from early logarithmic and late logarithmic/early stationary phase cultures, for the challenge experiments. The secondary culture of the relevant test organism was inoculated as normal and reference made to the appropriate growth curve to select suitable times for the above phases. At these times, sufficient test culture was filtered to obtain test suspensions containing about 5×10^7 cfu ml^{-1}. The standard challenge technique was then followed.

Figures 52-56 depict the results obtained for these experiments, and the $t_{0.1}$ values recorded are listed in Table 31.

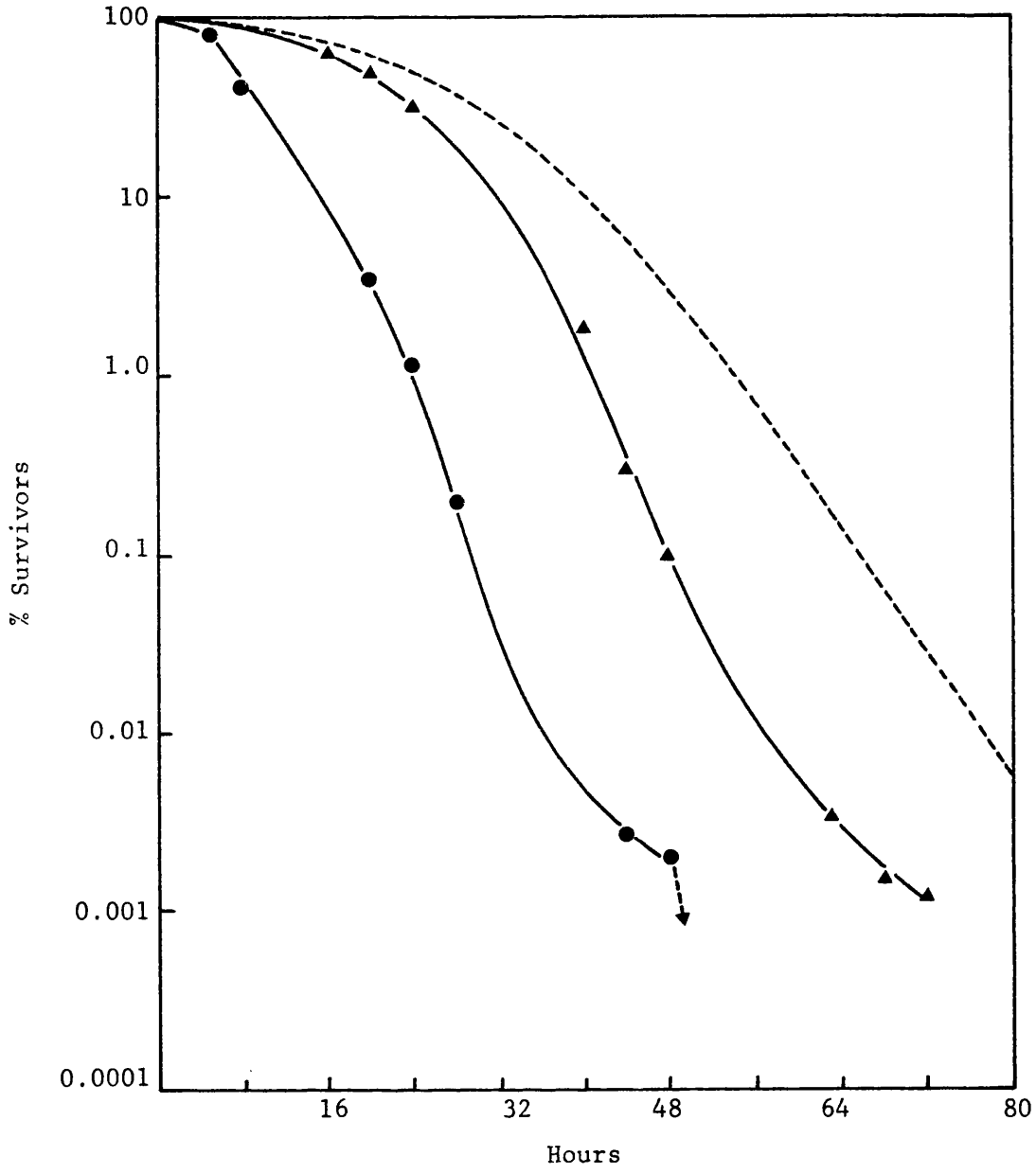


Fig.52 Effect of 0.008% w/v thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0, at 25°C on early logarithmic phase (●), late logarithmic/early stationary phase (▲) and late stationary phase (---) cells of Staph. aureus. Age of cells used: 4h, 10h and 22h respectively.

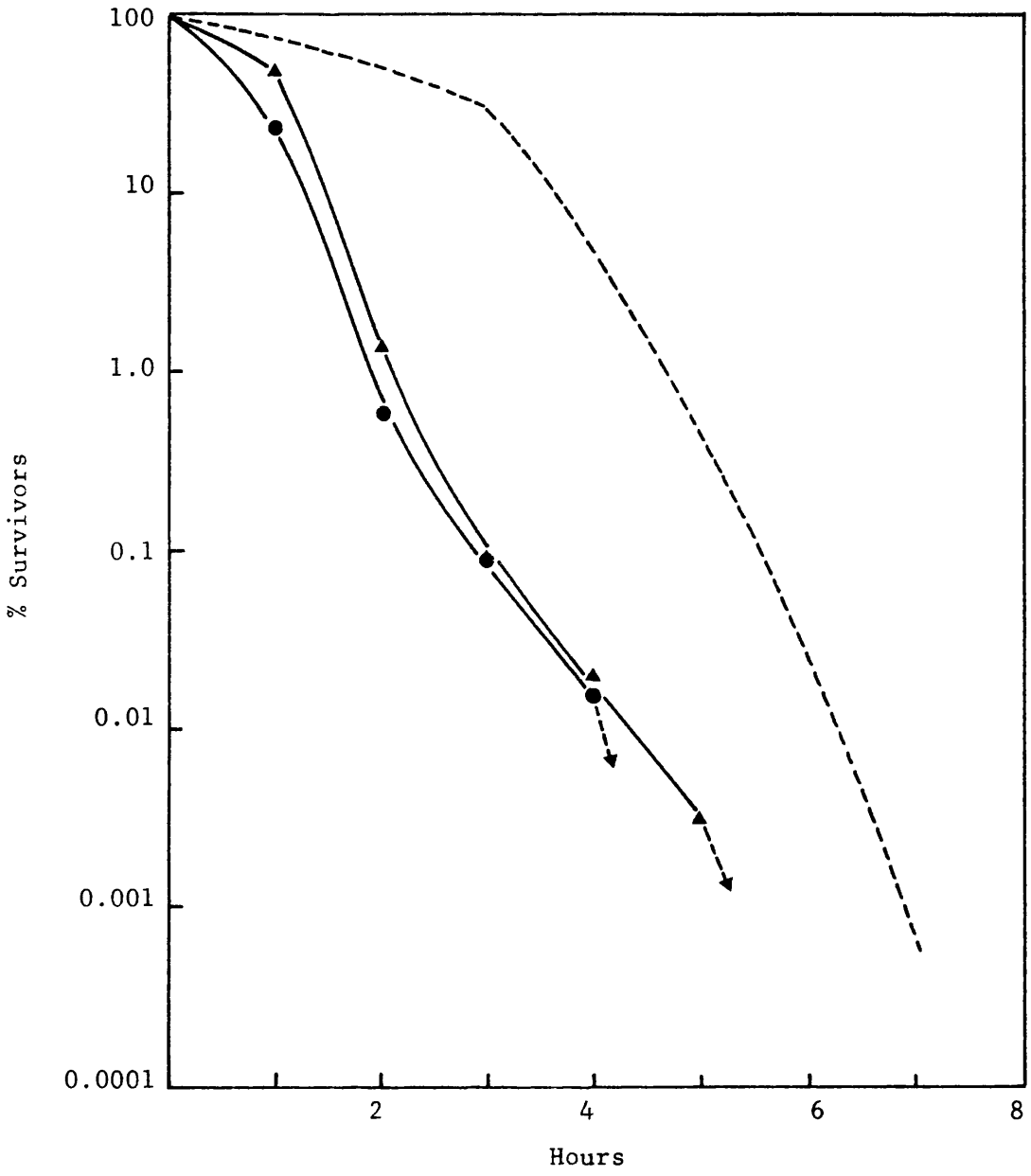


Fig.53 Effect of 0.008% w/v thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0 at 25°C on early logarithmic phase (●), late logarithmic/early stationary phase (▲) and late stationary phase (---) cells of Ps. aeruginosa. Age of cells used: 4h, 11h and 22h respectively.

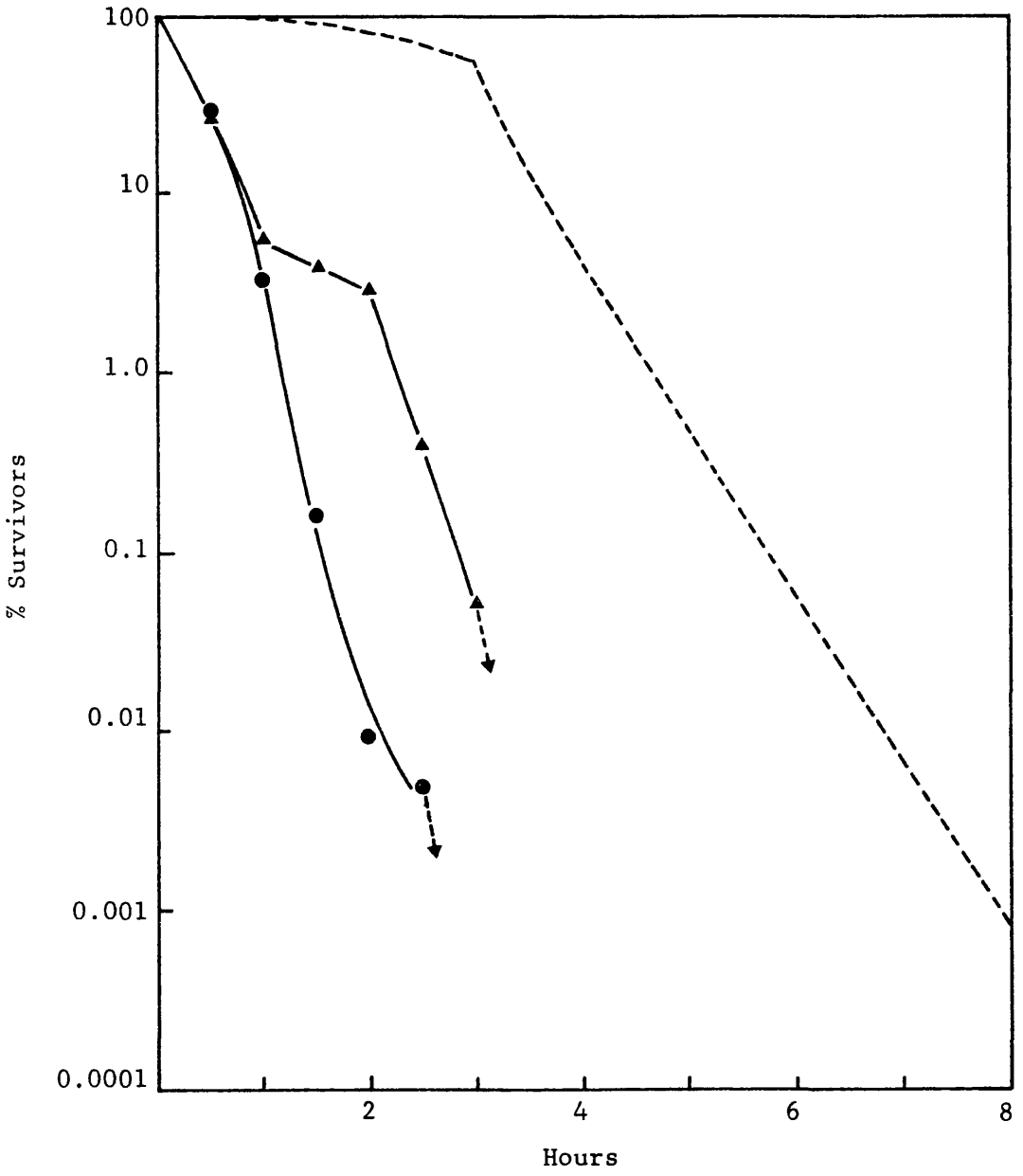


Fig.54 Effect of 0.008% w/v thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0, at 25°C on early logarithmic phase (●), late logarithmic/early stationary phase (▲) and late stationary phase (---) cells of E. coli. Age of cells used: 3h, 10h and 22h respectively.

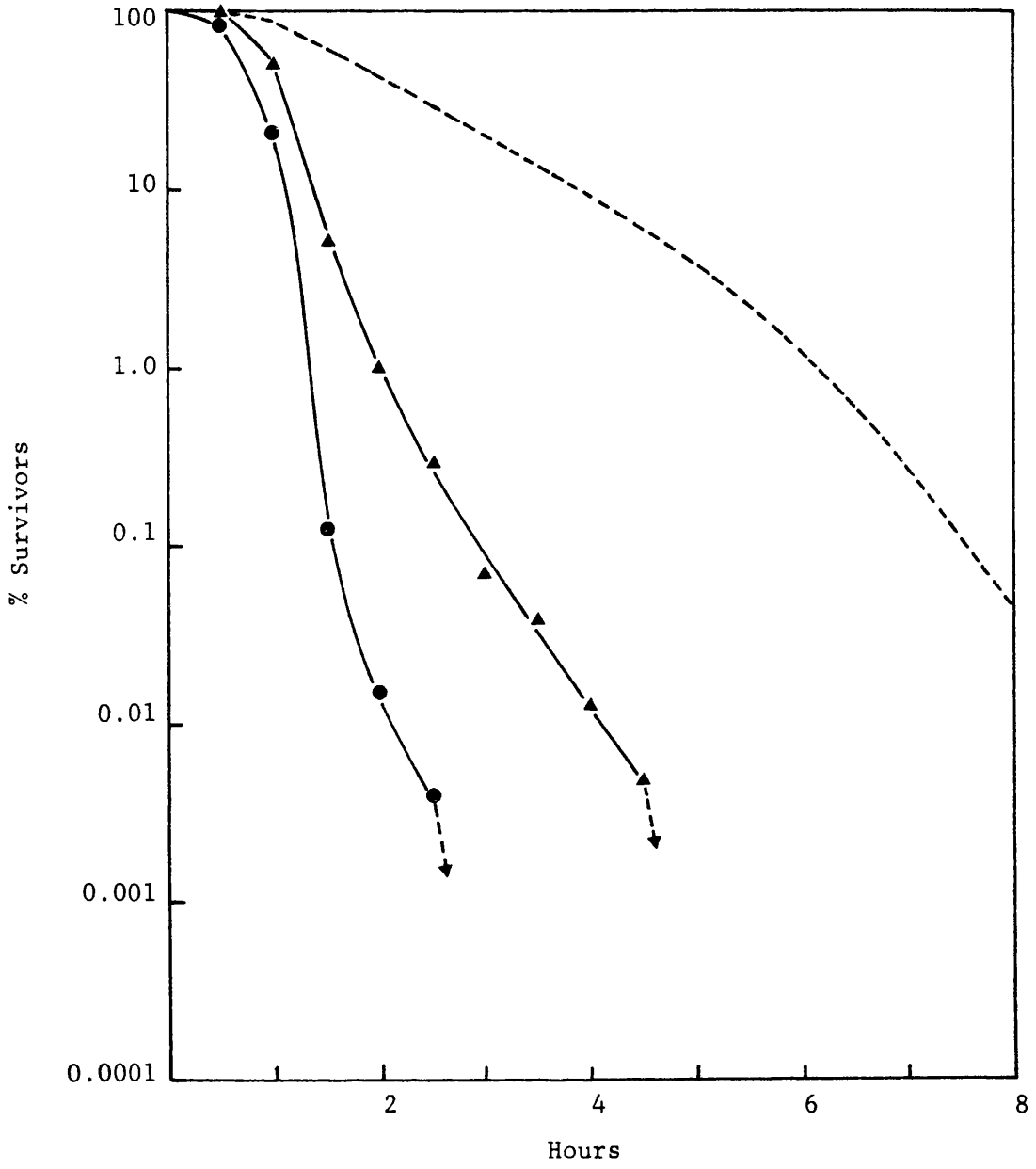


Fig.55 Effect of 0.008% w/v thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0, at 25°C on early logarithmic phase (●), late logarithmic/early stationary phase (▲) and late stationary phase (---) cells of C. albicans. Age of cells used: 3h, 10h and 22h respectively.

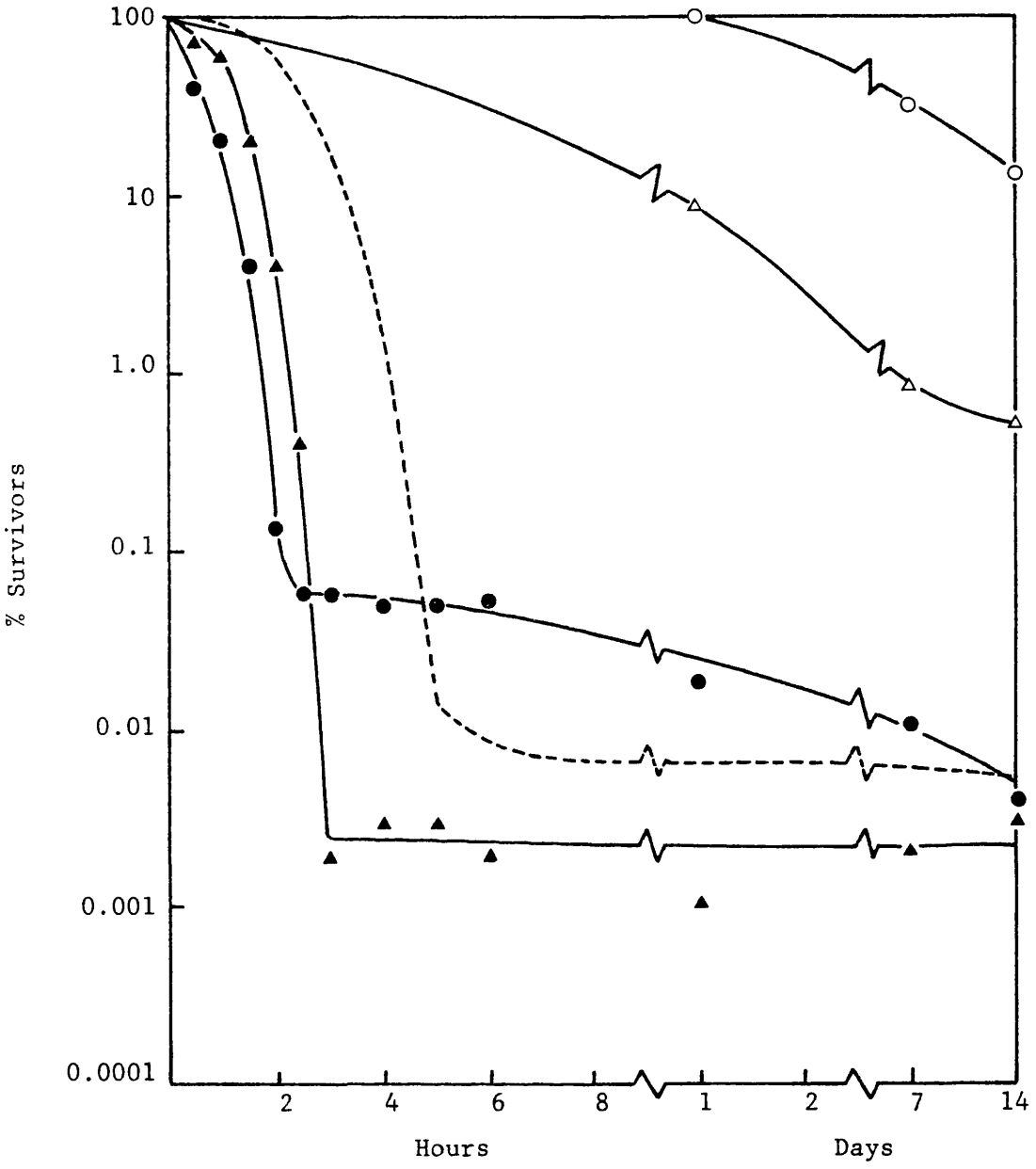


Fig.56 Effect of 0.008% w/v thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0, at 25°C on early logarithmic phase (●), late logarithmic/early stationary phase (▲) and late stationary phase (---) cells of B. subtilis. Age of cells used: 3h, 11h and 22h respectively.

Survival in buffer: early logarithmic phase (○) and late logarithmic/early stationary phase (△) cells

TABLE 31

$t_{0.1}$ values recorded from the effect of age of test culture on the antimicrobial activity of thiomersal.

Phase of Secondary Culture	$t_{0.1}$ values (hours)				
	Staph. aureus	Ps. aeruginosa	E. coli	C. albicans	B. subtilis
Early logarithmic	30.5	2.80	1.50	1.55	2.1
Late logarithmic/ Early stationary	48.0	3.05	2.85	2.90	2.6
Late stationary	66.5	5.55	5.95	7.40	4.4

8) Effect of Initial Inoculum Size

Sufficient microbial culture was filtered to give a stock suspension containing about 5×10^8 cfu ml^{-1}. Ten-fold serial dilutions were then made from this to give suspensions containing about 5×10^7, 5×10^6, 5×10^5 and 5×10^4 cfu ml^{-1}. These were in turn used to inoculate 5 test solutions of thiomersal, so that initial challenge sizes of 1×10^7, 1×10^6, 1×10^5, 1×10^4 and 1×10^3 cfu ml^{-1} were obtained. The 1×10^7 challenge level was omitted with C. albicans, as it was difficult to filter sufficient culture initially to obtain about 5×10^8 cfu ml^{-1}.

The results of these experiments are presented in Figures 57-61 and the $t_{0.1}$ values recorded are listed in Table 32.

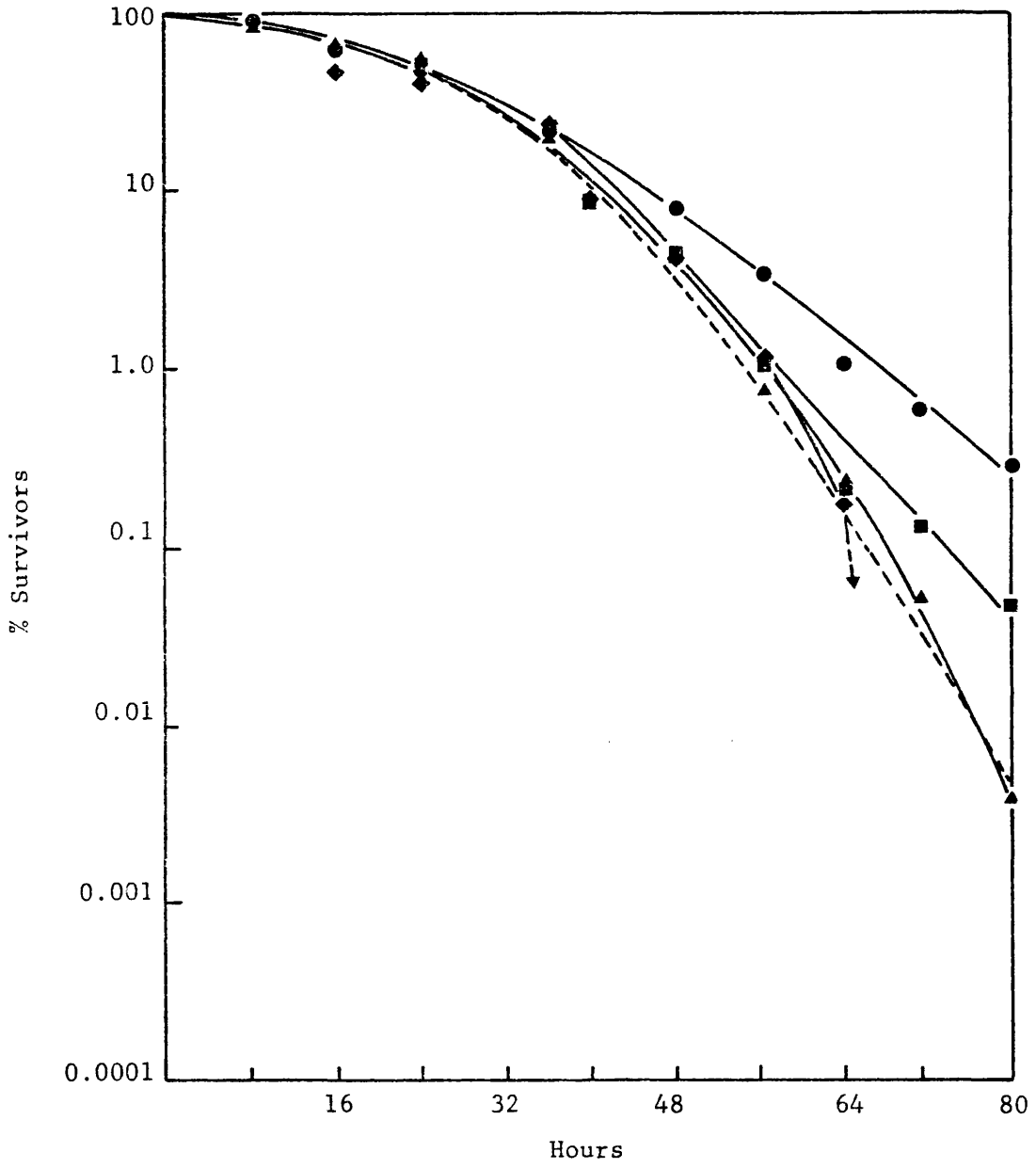


Fig.57 Effect of 0.008% w/v thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0, on inoculum sizes of 10^7 (●), 10^6 (---), 10^5 (▲), 10^4 (■) and 10^3 (◆) of Staph. aureus.

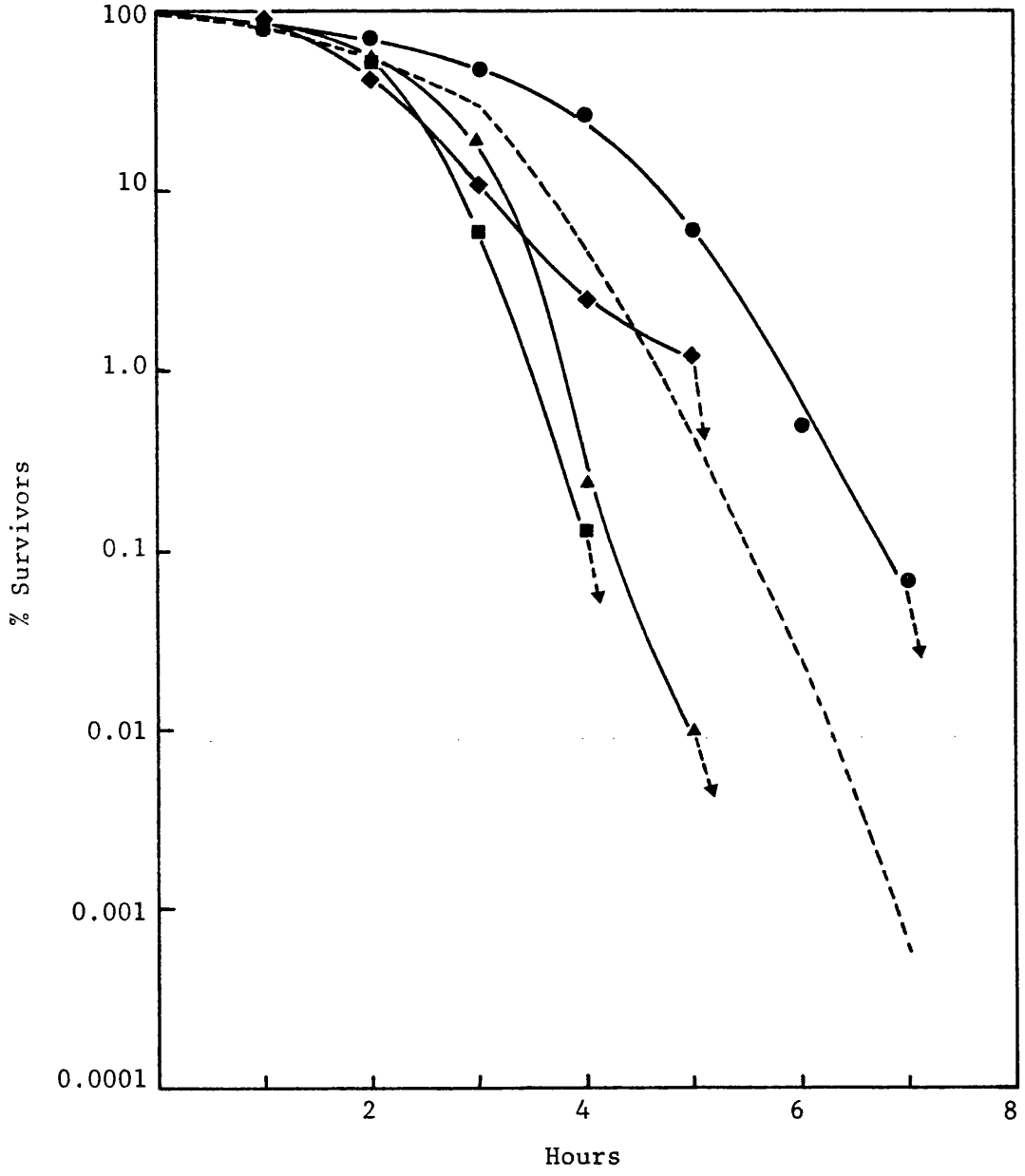


Fig.58 Effect of 0.008% w/v thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0, on inoculum sizes of 10^7 (●), 10^6 (---), 10^5 (▲), 10^4 (■) and 10^3 (◆) of Ps. aeruginosa.

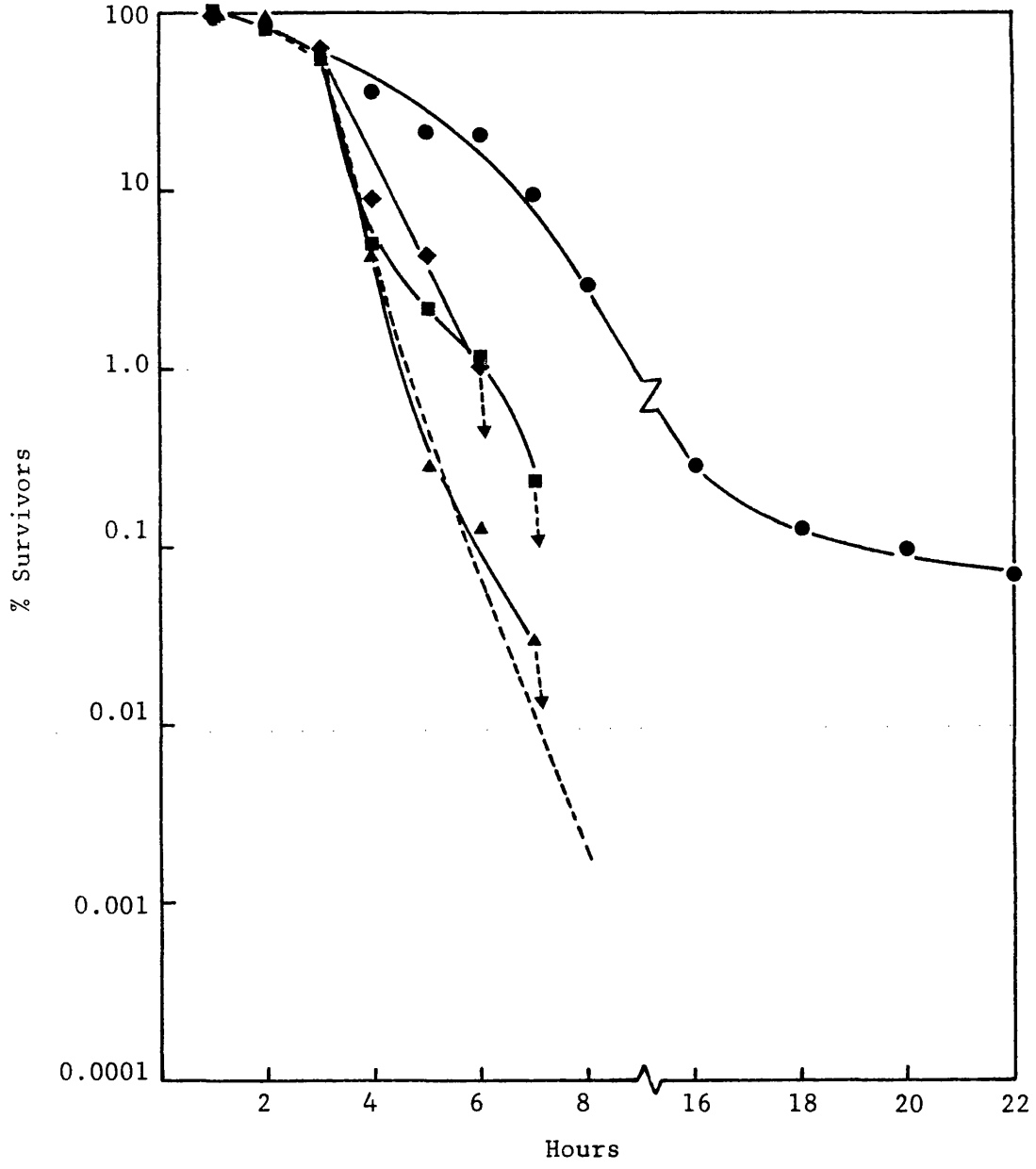


Fig.59 Effect of 0.008% w/v thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0, on inoculum sizes of 10^7 (●), 10^6 (---), 10^5 (▲), 10^4 (■) and 10^3 (◆) of E. coli.

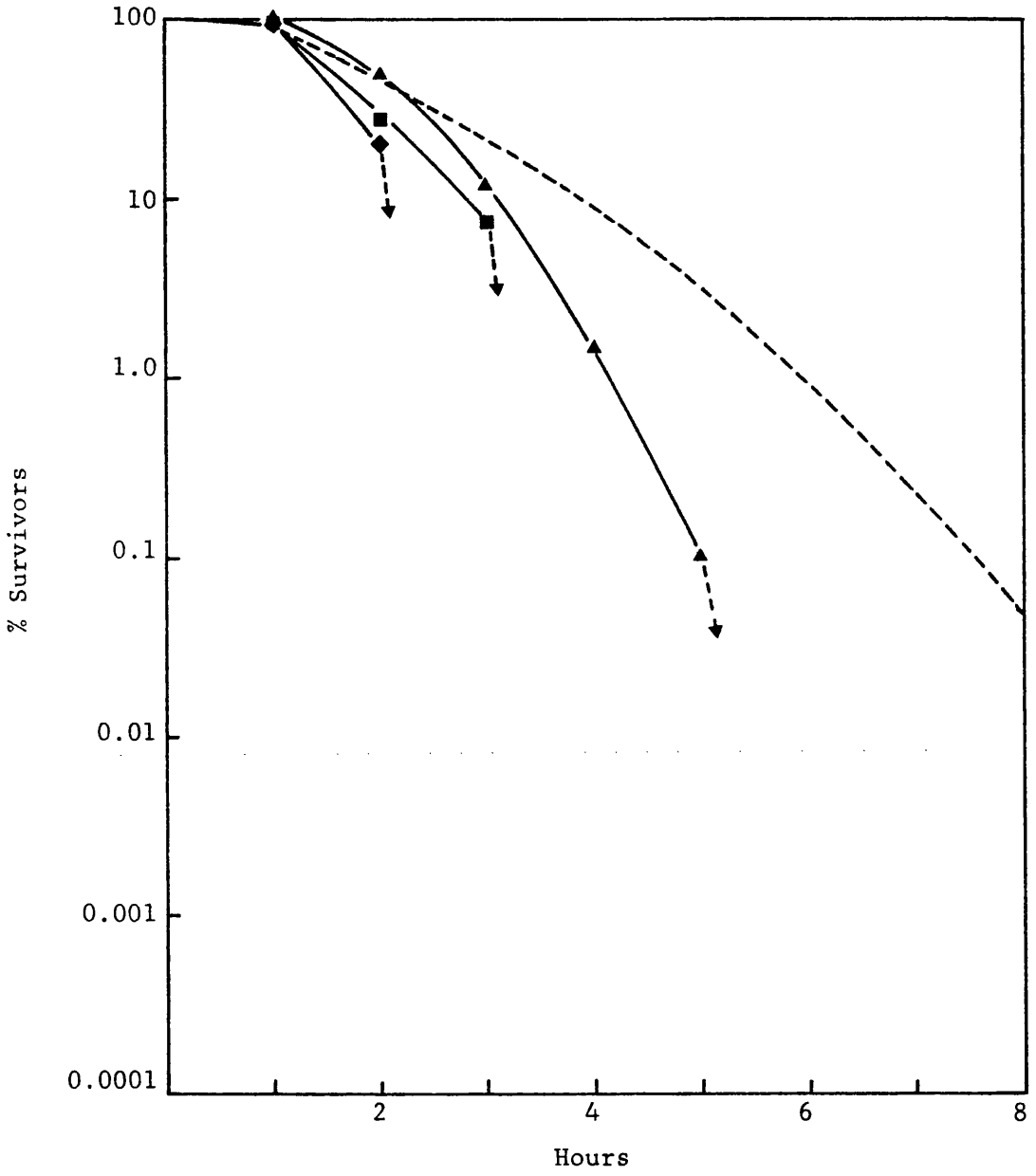


Fig.60 Effect of 0.008% w/v thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0, on inoculum sizes of 10^6 (---), 10^5 (▲), 10^4 (■) and 10^3 (◆) of C. albicans.

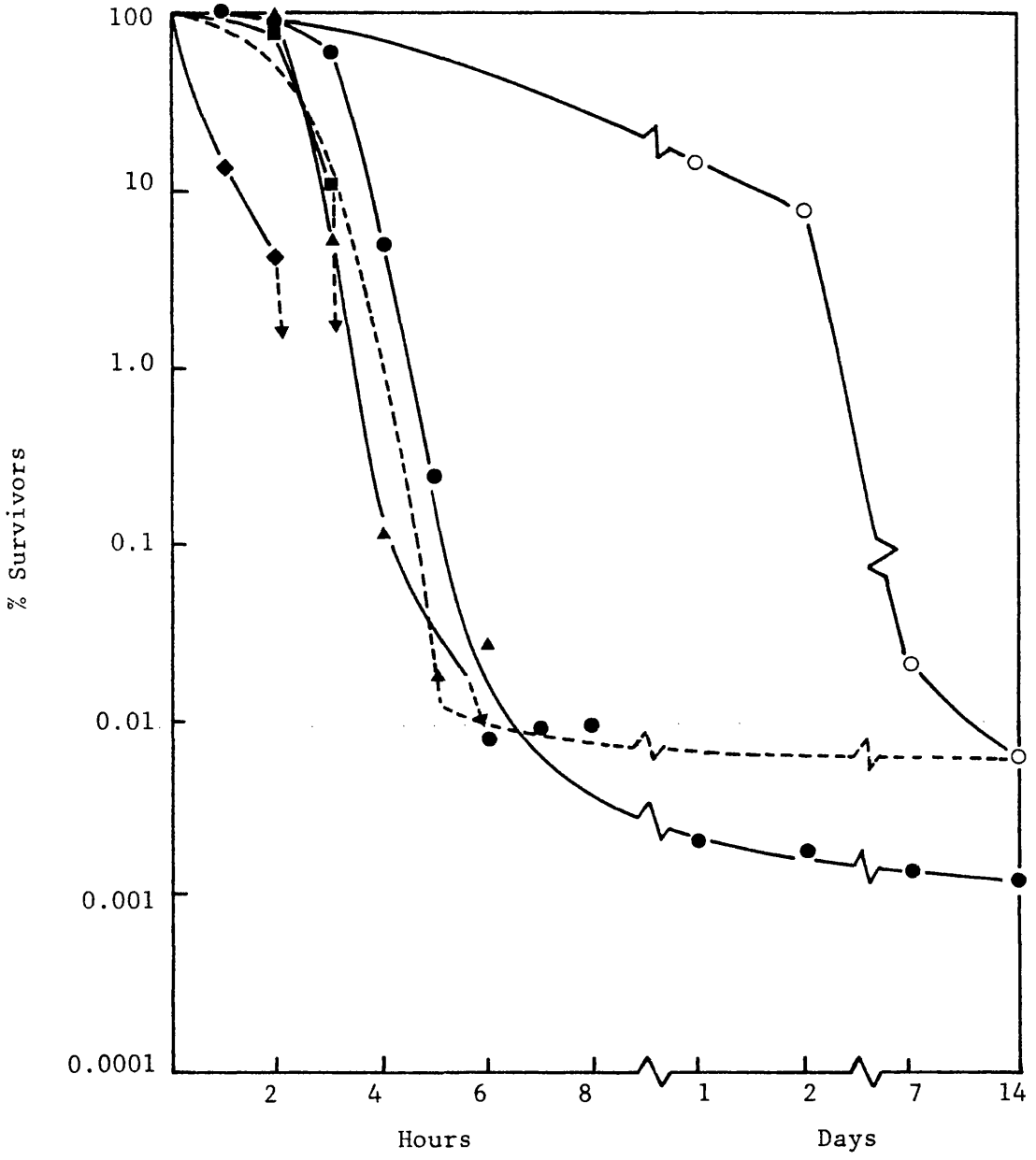


Fig.61 Effect of 0.008% w/v thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0, on inoculum sizes of 10^7 (●), 10^6 (---), 10^5 (▲), 10^4 (■) and 10^3 (◆) of B. subtilis.

(○) Survival of 10^7 cfu ml^{-1} in buffer at 25°C

TABLE 32

$^{+}_{+}t_{0.1}$ values recorded from the effect of initial inoculum size on the antimicrobial activity of thiomersal.

Initial Inoculum size (cfu ml^{-1})	$^{+}_{+}t_{0.1}$ values (hours)				
	Staph. aureus	Ps. aeruginosa	E. coli	C. albicans	B. subtilis
1×10^{7}	67.0	6.80	19.10	N.D.	5.2
1×10^{6}	54.5	5.55	5.95	7.55	4.4
1×10^{5}	56.0	4.25	6.0	5.00	4.1
1×10^{4}	57.0	4.05	7-8	3-4	3-4
1×10^{3}	56.0	5-6	6-7	2-3	2-3

$^{+}_{+}t_{1.0}$ values read for Staph. aureus

N.D. Not Done (see page 174)

EFFECT OF PHOTOCHEMICAL DEGRADATION ON THE ANTIMICROBIAL ACTIVITY OF THIOMERSAL

1) Test Solutions Prepared in Phosphate Buffer

Undegraded and 2, 4, 6, 8 and 10-day photochemically degraded solutions, obtained as described on page 88, were used for these experiments. An initial inoculum size of 1×10^6 cfu ml^{-1} was prepared and the standard technique followed. The ability of the recovery medium to inactivate degraded solutions and allow surviving organisms to grow, had previously been determined.

Figures 62-67 represent the results obtained from these experiments. As degraded solutions seem to have a marked effect on Ps. aeruginosa, a further strain of this organism, namely, Ps. aeruginosa NCTC 6749, was included in the challenge series. Figure 64 depicts the results obtained with this strain. The $t_{0.1}$ values obtained from these challenge experiments are listed in Table 33.

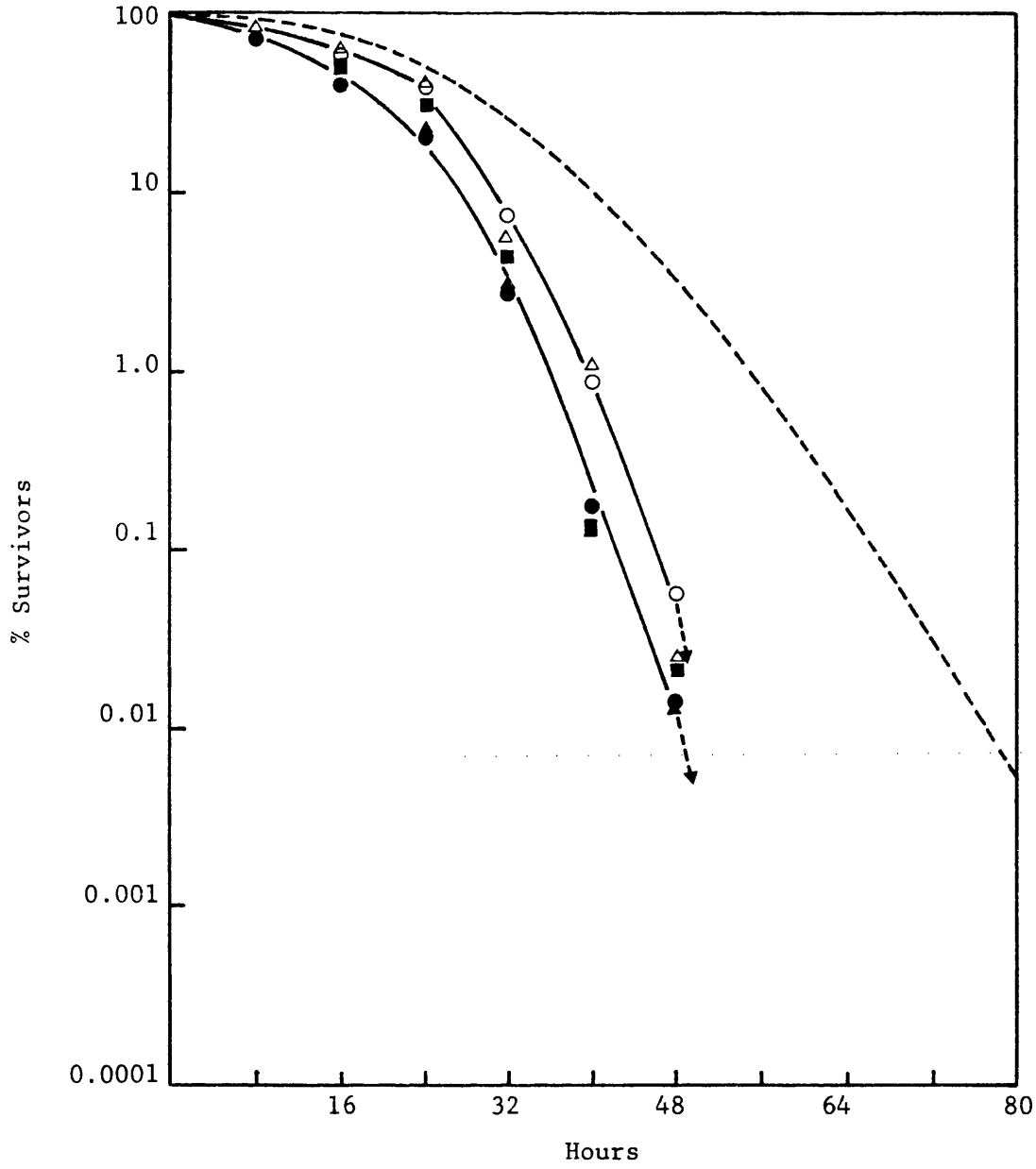


Fig.62 Effect of undegraded (---), 2-day (●), 4-day (▲), 6-day (■), 8-day (○) and 10-day (△) degraded solutions of thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0 at 25°C, on Staph. aureus.

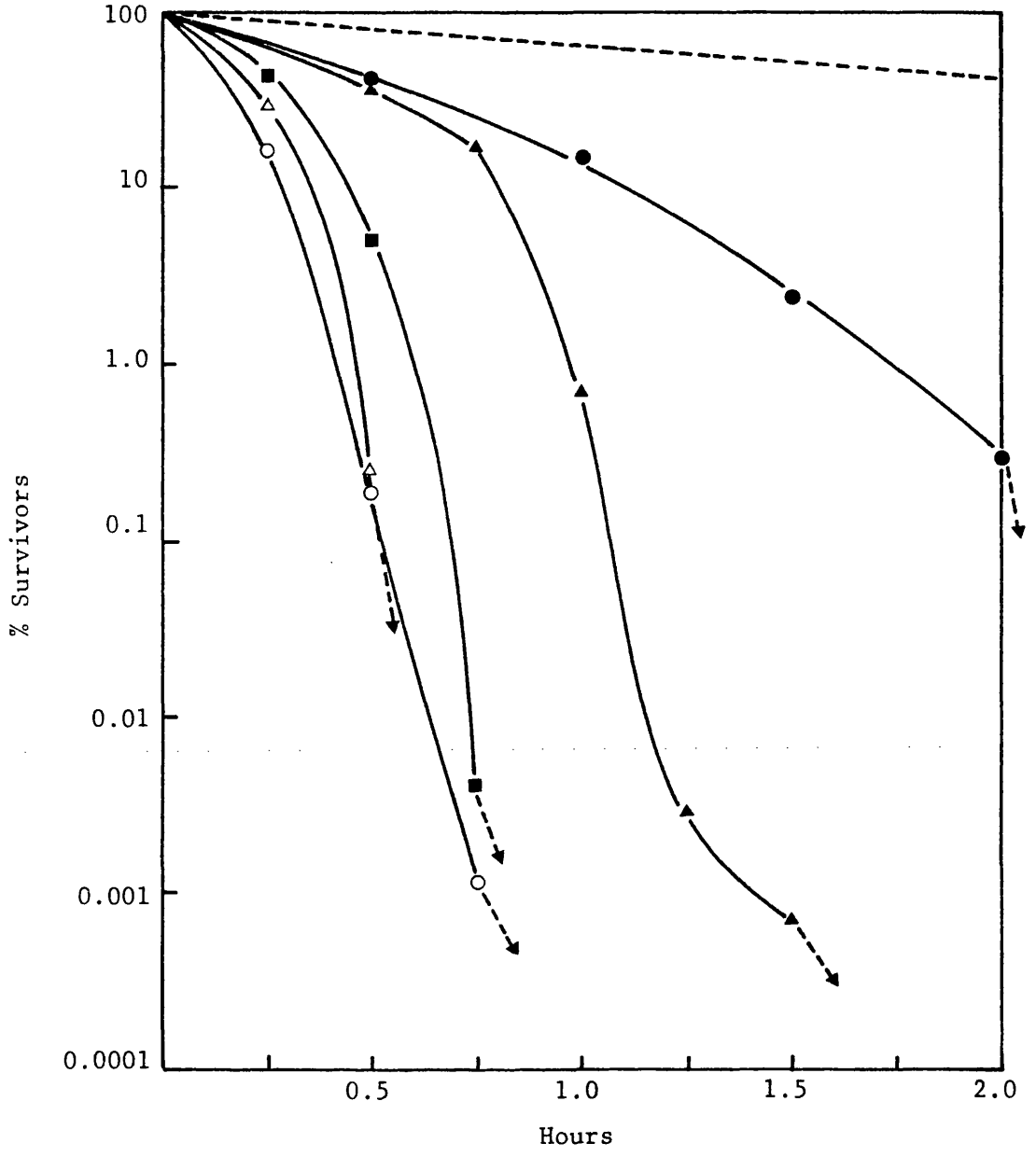


Fig.63 Effect of undegraded (---), 2-day (●), 4-day (▲), 6-day (■), 8-day (○) and 10-day (△) degraded solutions of thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0, at 25°C, on Ps. aeruginosa NCTC 6750.

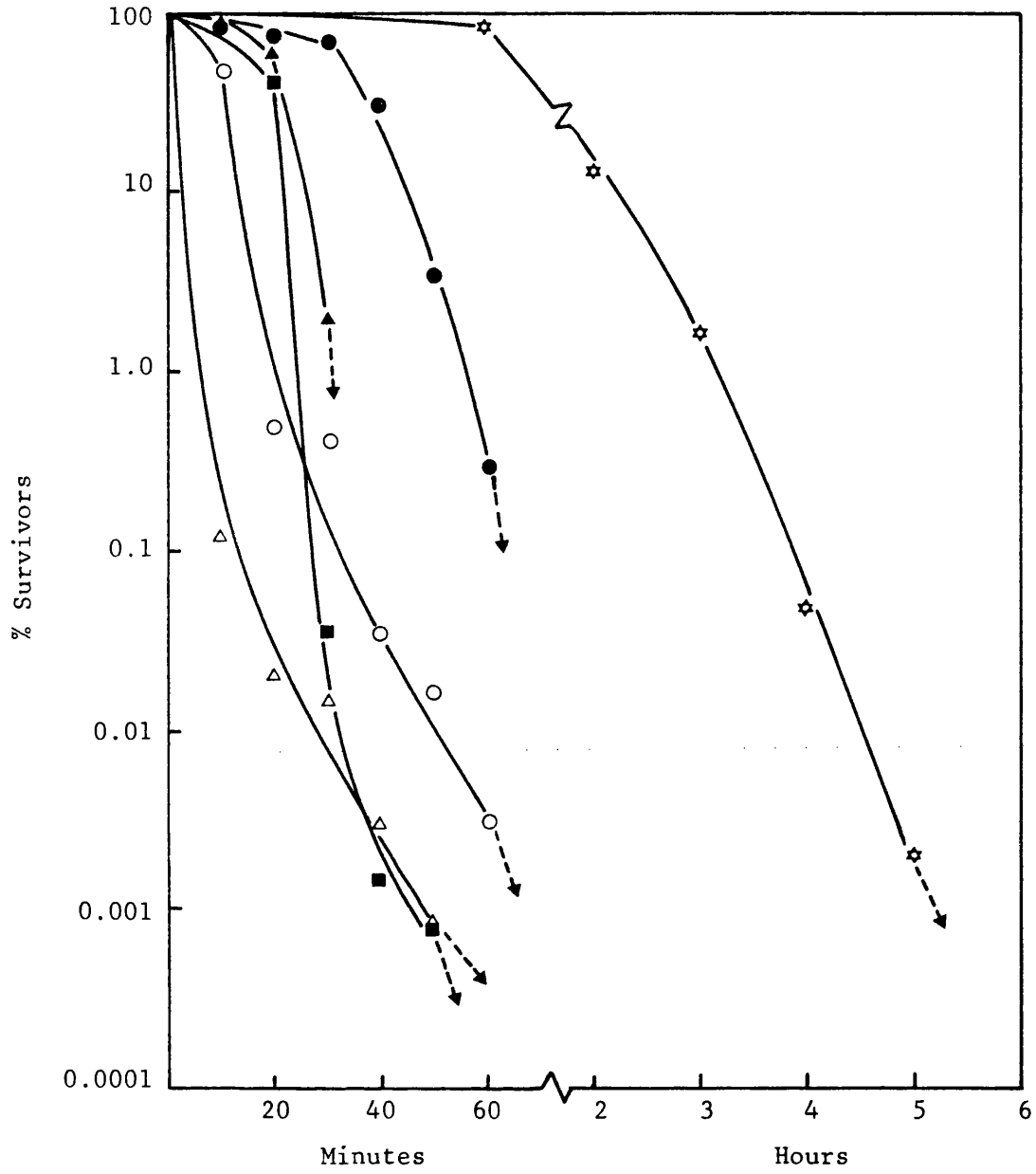


Fig.64 Effect of undegraded (♦), 2-day (●), 4-day (▲), 6-day (■), 8-day (○) and 10-day (△) degraded solutions of thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0, at 25°C, on Ps. aeruginosa NCTC 6749.

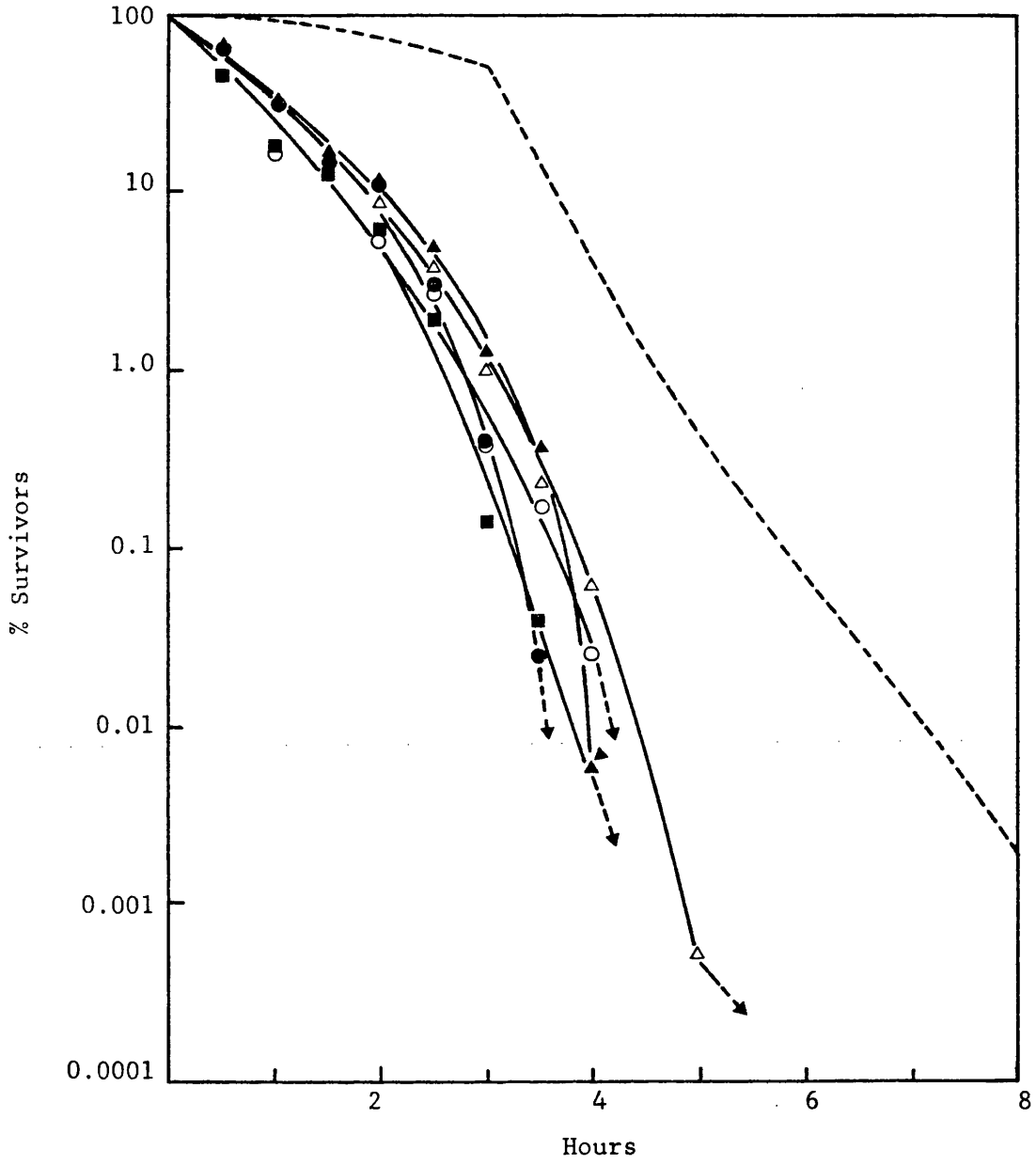


Fig.65 Effect of undegraded (---), 2-day (●), 4-day (▲), 6-day (■), 8-day (○) and 10-day (△) degraded solutions of thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0, at 25°C, on E.coli.

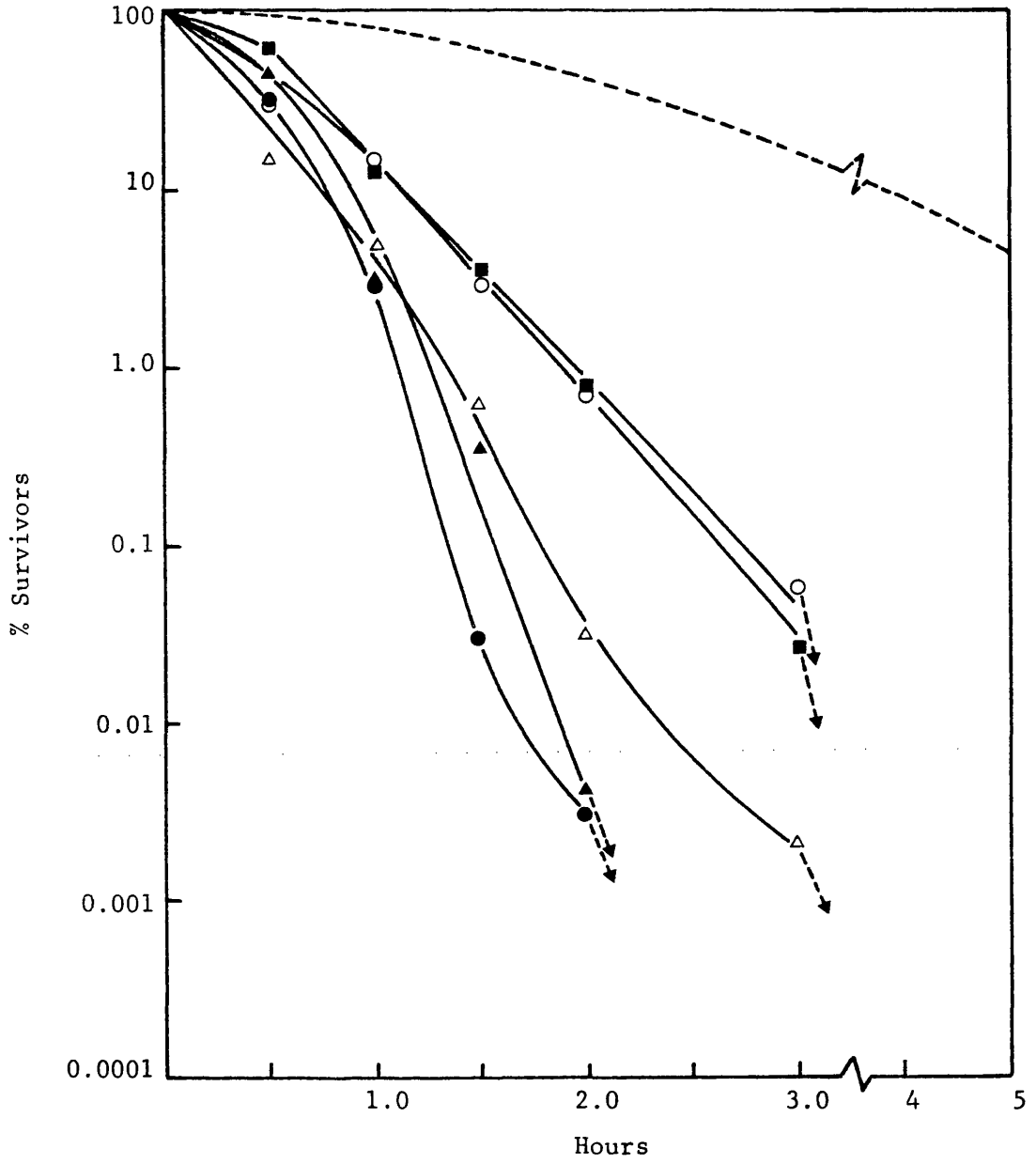


Fig.66 Effect of undegraded (---), 2-day (●), 4-day (▲), 6-day (■), 8-day (○) and 10-day (△) degraded solutions of thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0, at 25°C, on C. albicans.

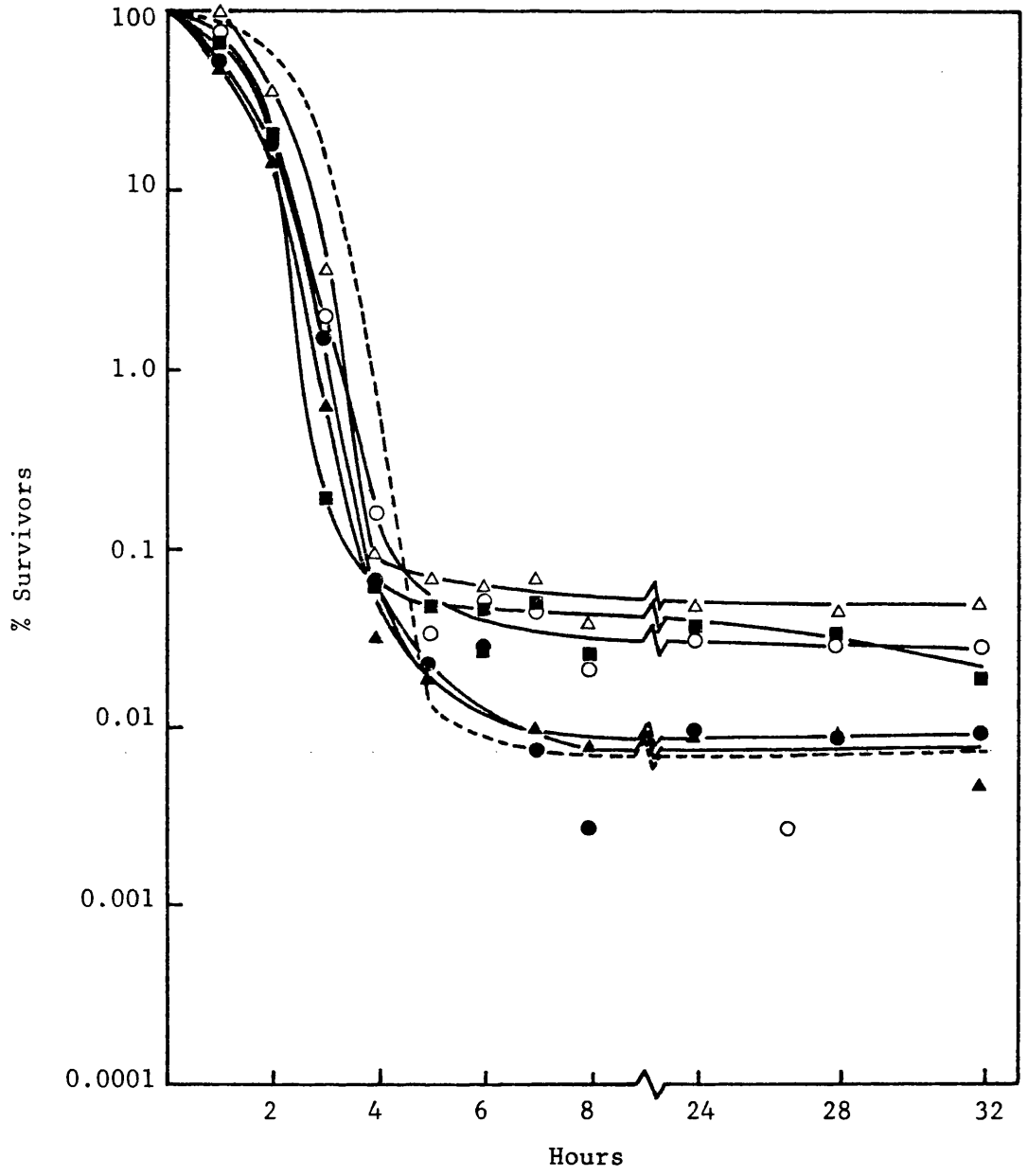


Fig.67 Effect of undegraded (---), 2-day (●), 4-day (▲), 6-day (■), 8-day (○) and 10-day (△) degraded solutions of thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0, at 25°C, on B. subtilis.

TABLE 33

‡‡$t_{0.1}$ values recorded from the antimicrobial acitvity of photochemically degraded solutions of thiomersal in buffer.

Time in Light box (days)	$t_{0.1}$ values (hours) Staph. aureus	‡‡Ps. aeruginosa NCTC 6750	‡‡Ps. aeruginosa NCTC 6749	E. coli	C. albicans	B. subtilis
0	66.5	4.70	3.10	5.95	7.55	4.4
2	41.5	1.75	0.85	3.25	1.37	3.9
4	41.5	0.90	0.50-0.67	3.70	1.53	3.5
6	41.5	0.62	0.37	3.30	2.60	3.5
8	45.5	0.43	0.35	3.60	2.80	4.3
10	45.5	0.45	0.08	3.90	1.80	4.0

‡‡$t_{1.0}$ values read for these organisms.

2) Test Solutions Prepared in Phosphate Buffer with 0.01% w/v EDTA

Undegraded and 2, 4, 6, 8 and 10-day degraded solutions in isotonic Sörensen's phosphate buffer, pH 7.0, with 0.01% w/v EDTA were used in these experiments. The tests were carried out using the standard challenge technique.

Figures 68-72 represent the results obtained and the $t_{0.1}$ values recorded from these experiments are presented in Table 34.

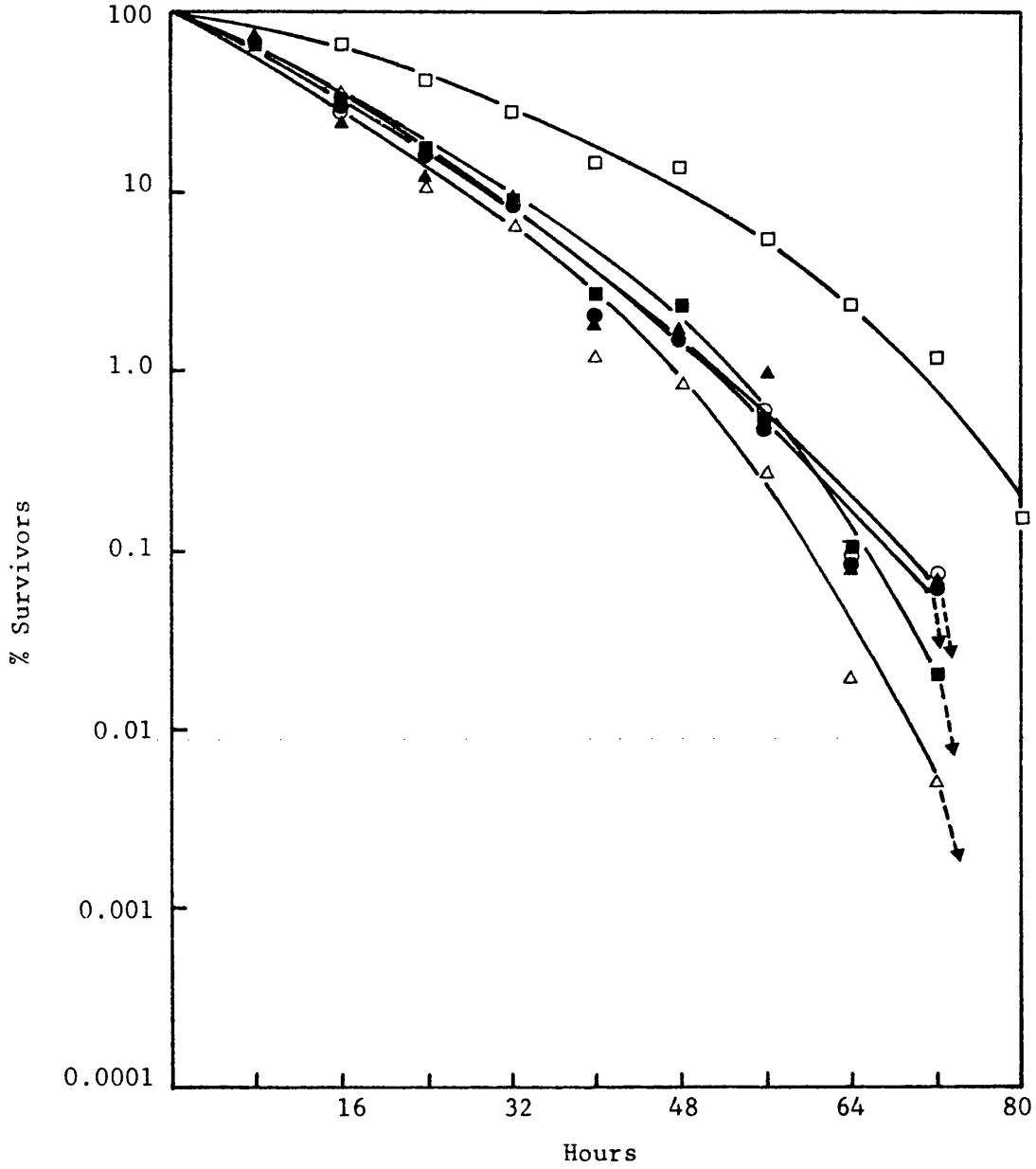


Fig.68 Effect of undegraded (□), 2-day (●), 4-day (▲), 6-day (■), 8-day (○) and 10-day (△) degraded solutions of thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0, with 0.01% w/v EDTA, at 25°C, on Staph. aureus.

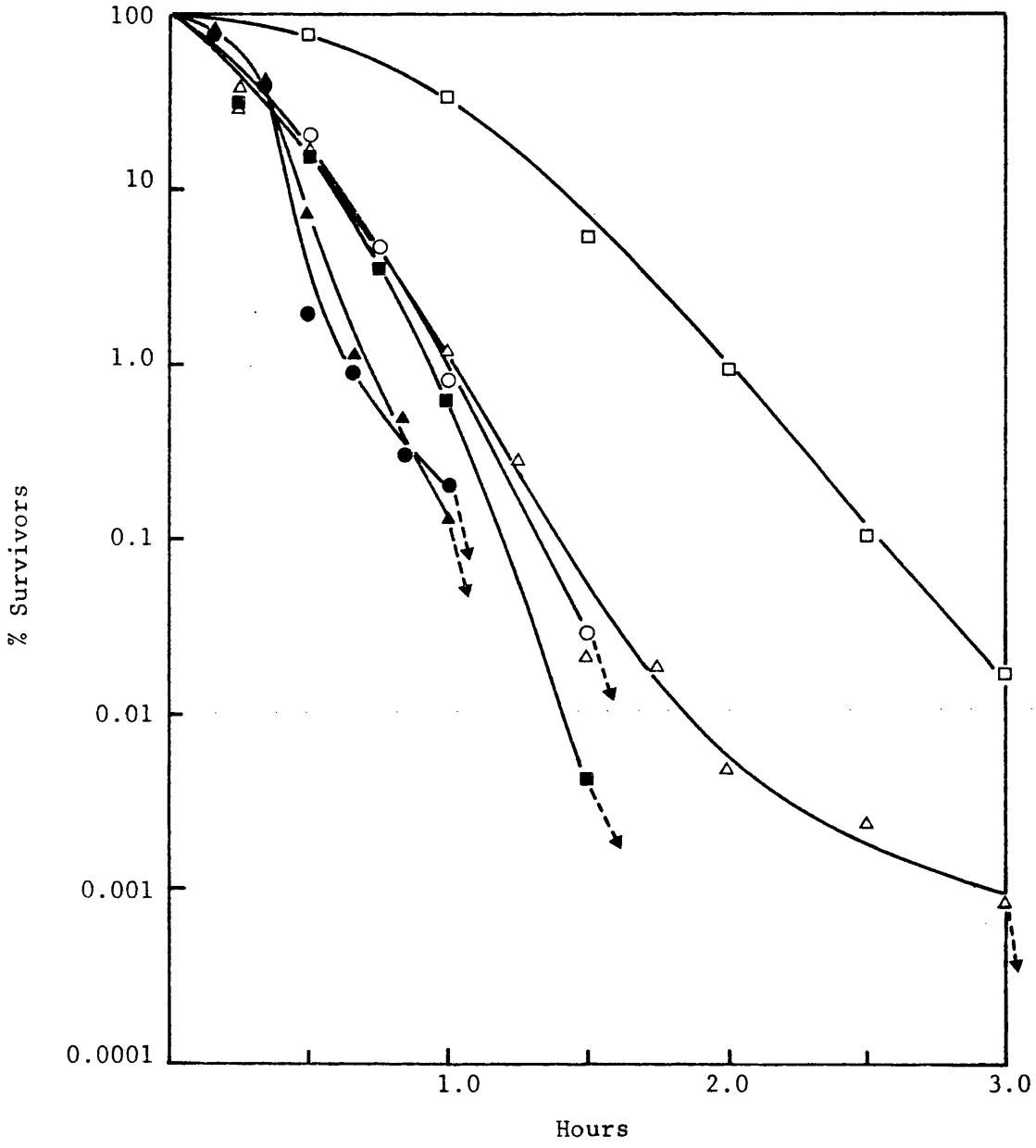


Fig.69 Effect of undegraded (□), 2-day (●), 4-day (▲), 6-day (■), 8-day (○) and 10-day (△) degraded solutions of thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0, with 0.01% w/v EDTA, at 25°C, on Ps. aeruginosa.

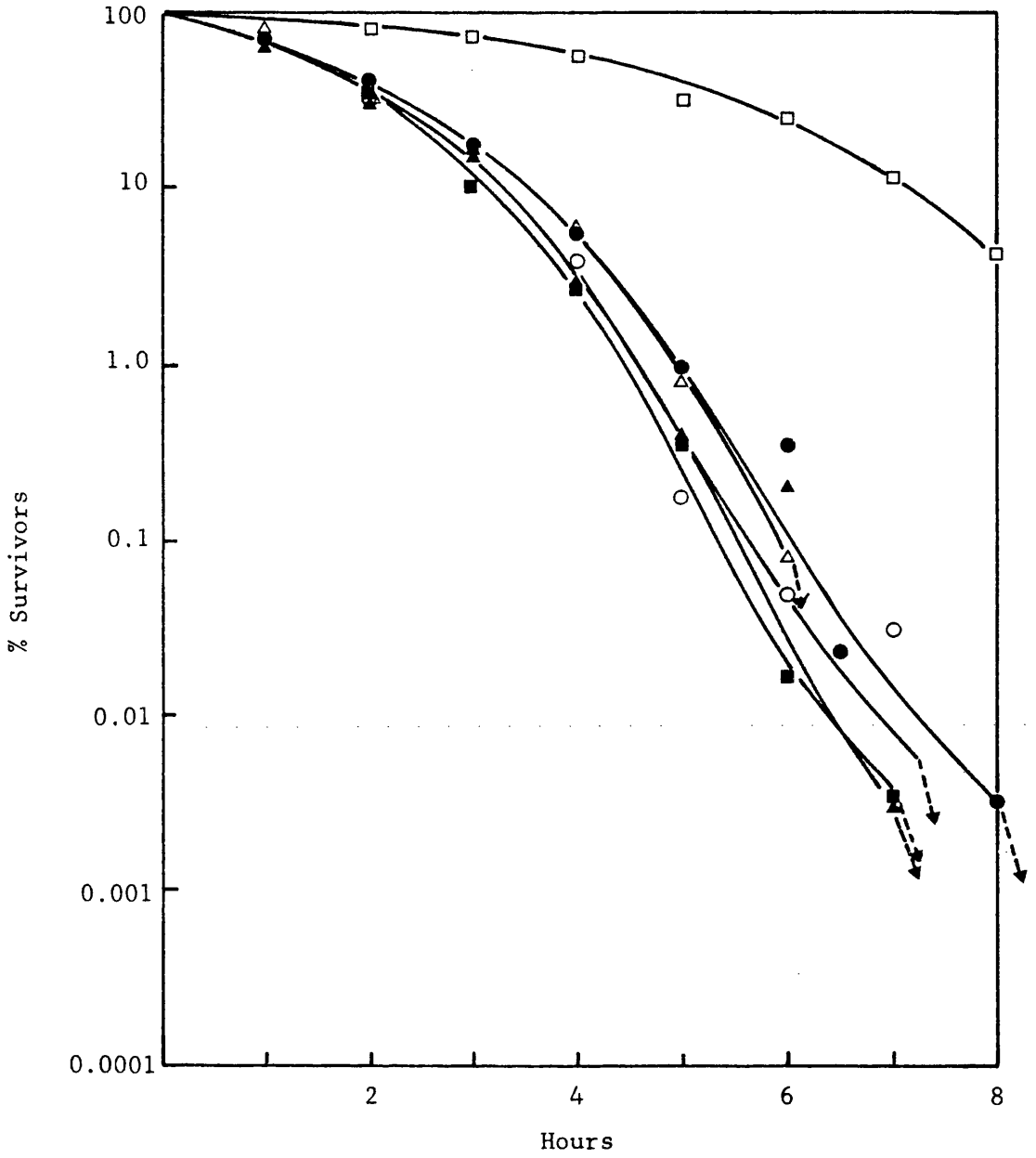


Fig.70 Effect of undegraded (□), 2-day (●), 4-day (▲), 6-day (■), 8-day (○) and 10-day (△) degraded solutions of thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0, with 0.01% w/v EDTA, at 25°C, on E. coli.

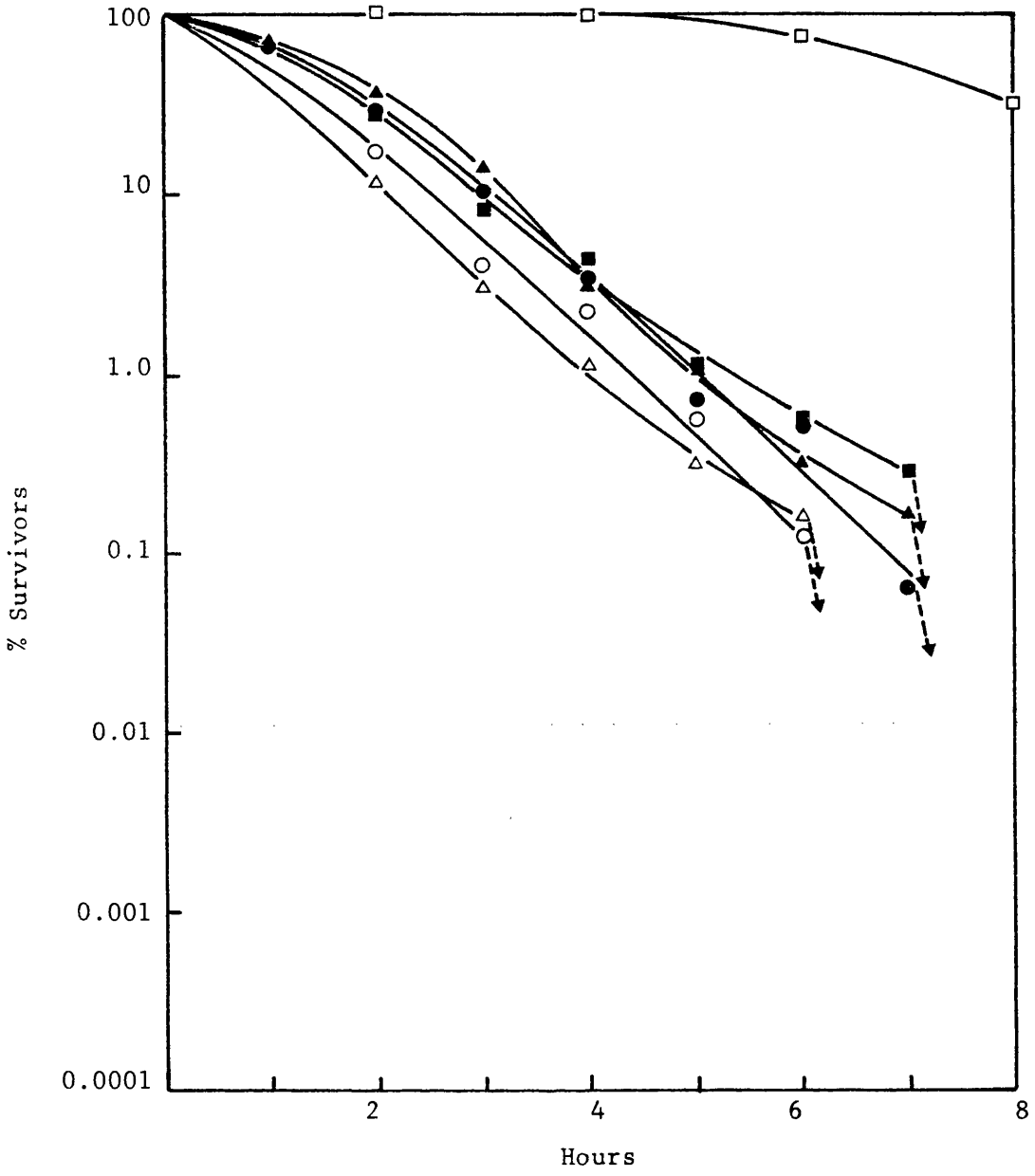


Fig.71 Effect of undegraded (□), 2-day (●), 4-day (▲), 6-day (■), 8-day (○) and 10-day (△) degraded solutions of thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0, with 0.01% w/v EDTA, at 25°C, on C. albicans.

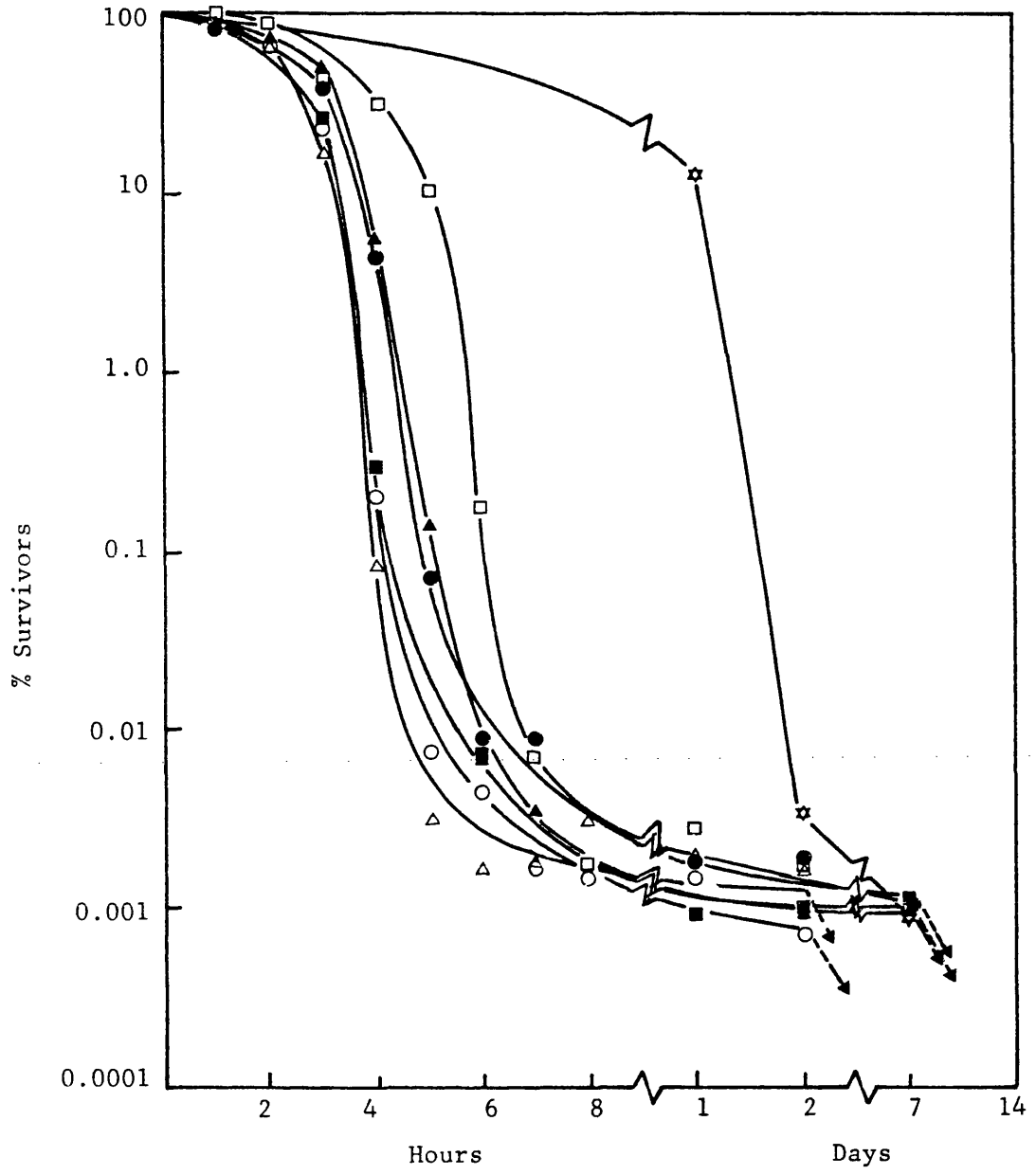


Fig.72 Effect of undegraded (□), 2-day (●), 4-day (▲), 6-day (■), 8-day (○) and 10-day (△) degraded solutions of thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0, with 0.01% w/v EDTA, at 25°C, on B. subtilis.

(✡) survival in buffer with 0.01% w/v EDTA

TABLE 34

‡‡$t_{0.1}$ values recorded from the antimicrobial activity of photochemically degraded solutions of thiomersal in buffer with 0.01% w/v EDTA.

Time in Light box (Days)	$t_{0.1}$ values (hours) ‡‡Staph. aureus	‡‡Ps. aeruginosa	E. coli	‡‡C. albicans	B. subtilis
0	71.0	1.98	8-16	16.8	5.1
2	49.5	0.67	6.05	5.0	3.9
4	49.5	0.72	5.70	5.0	3.9
6	53.0	0.95	5.45	5.2	3.9
8	49.5	0.98	5.70	4.4	3.7
10	47.5	1.03	5.90	4.0	4.6

‡‡$t_{1.0}$ values read for these organisms

3) Test Solutions Prepared in Phosphate Buffer with 0.1% w/v EDTA

Undegraded and 2, 4, 6, 8 and 10-day degraded solutions in isotonic Sörensen's phosphate buffer, pH 7.0, with 0.1% w/v EDTA were used in this series of experiments. The standard challenge technique was followed here too.

Figures 73-77 depict the results obtained and the $t_{0.1}$ values are listed in Table 35.

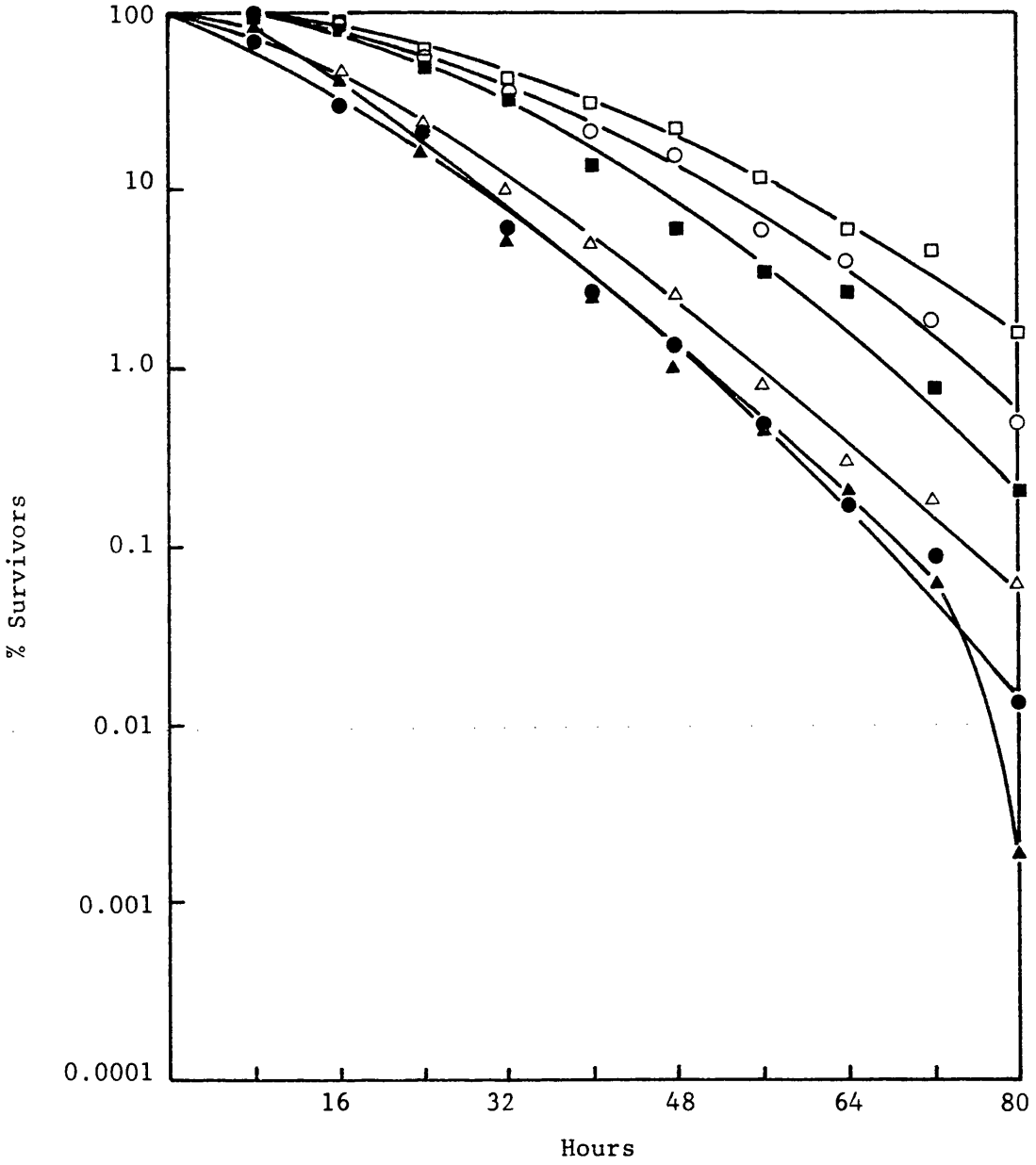


Fig.73 Effect of undegraded (□), 2-day (●), 4-day (▲), 6-day (■), 8-day (○) and 10-day (△) degraded solutions of thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0, with 0.1% w/v EDTA, at 25°C, on Staph. aureus.

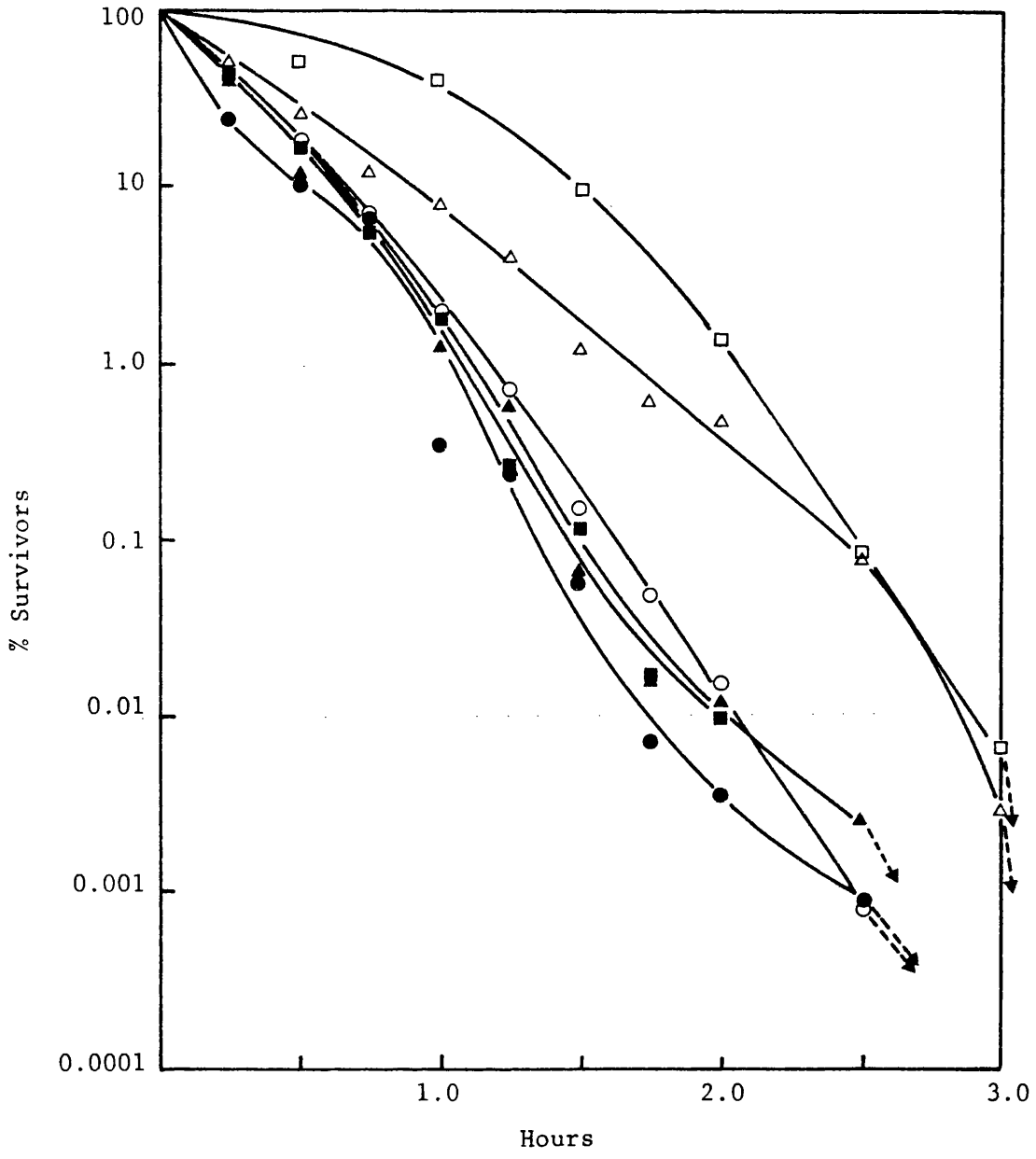


Fig.74 Effect of undegraded (□), 2-day (●), 4-day (▲), 6-day (■), 8-day (○) and 10-day (△) degraded solutions of thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0, with 0.1% w/v EDTA, at 25°C, on Ps. aeruginosa.

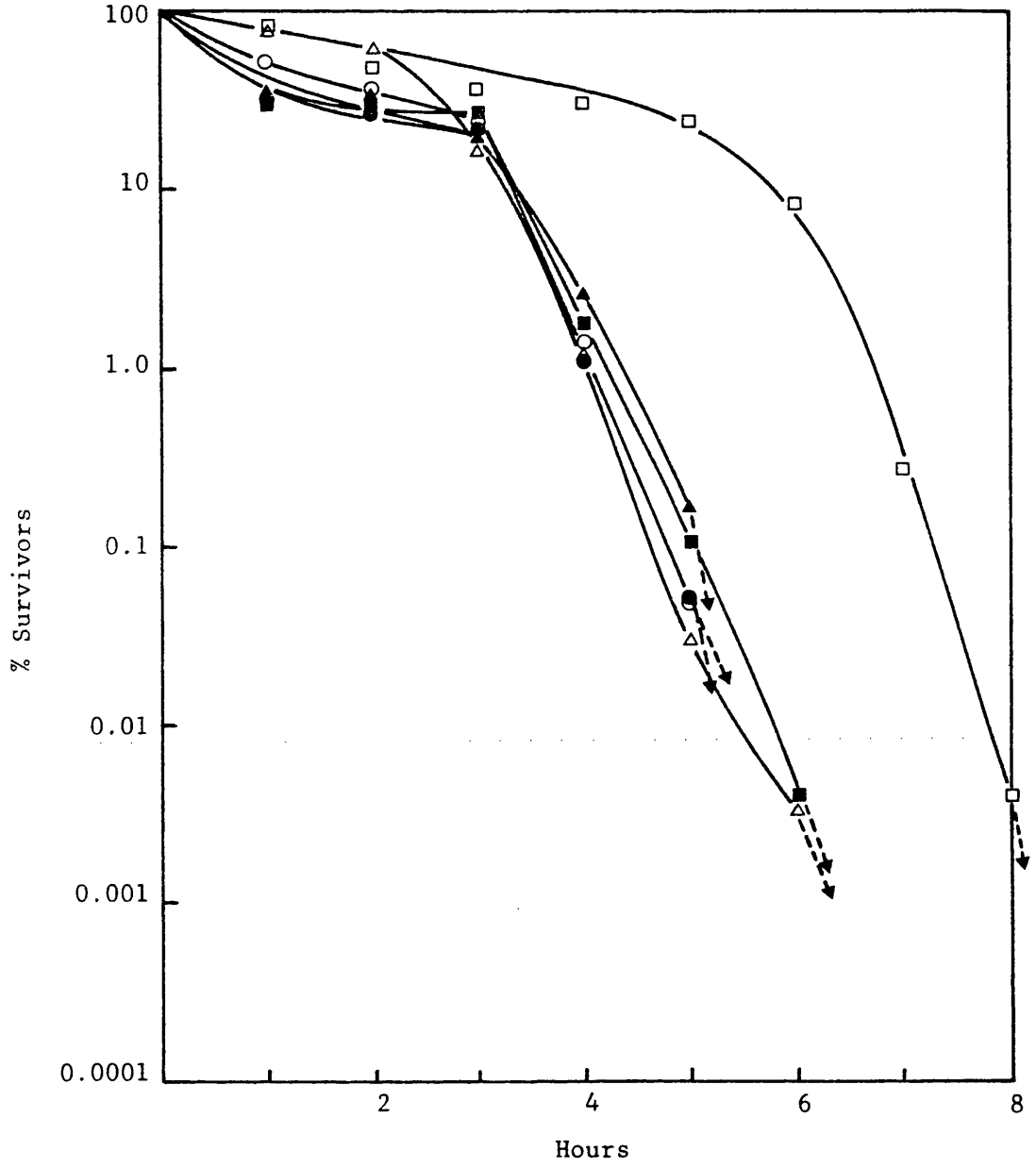


Fig.75 Effect of undegraded (□), 2-day (●), 4-day (▲), 6-day (■), 8-day (○) and 10-day (△) degraded solutions of thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0, with 0.1% w/v EDTA, at 25°C, on E. coli.

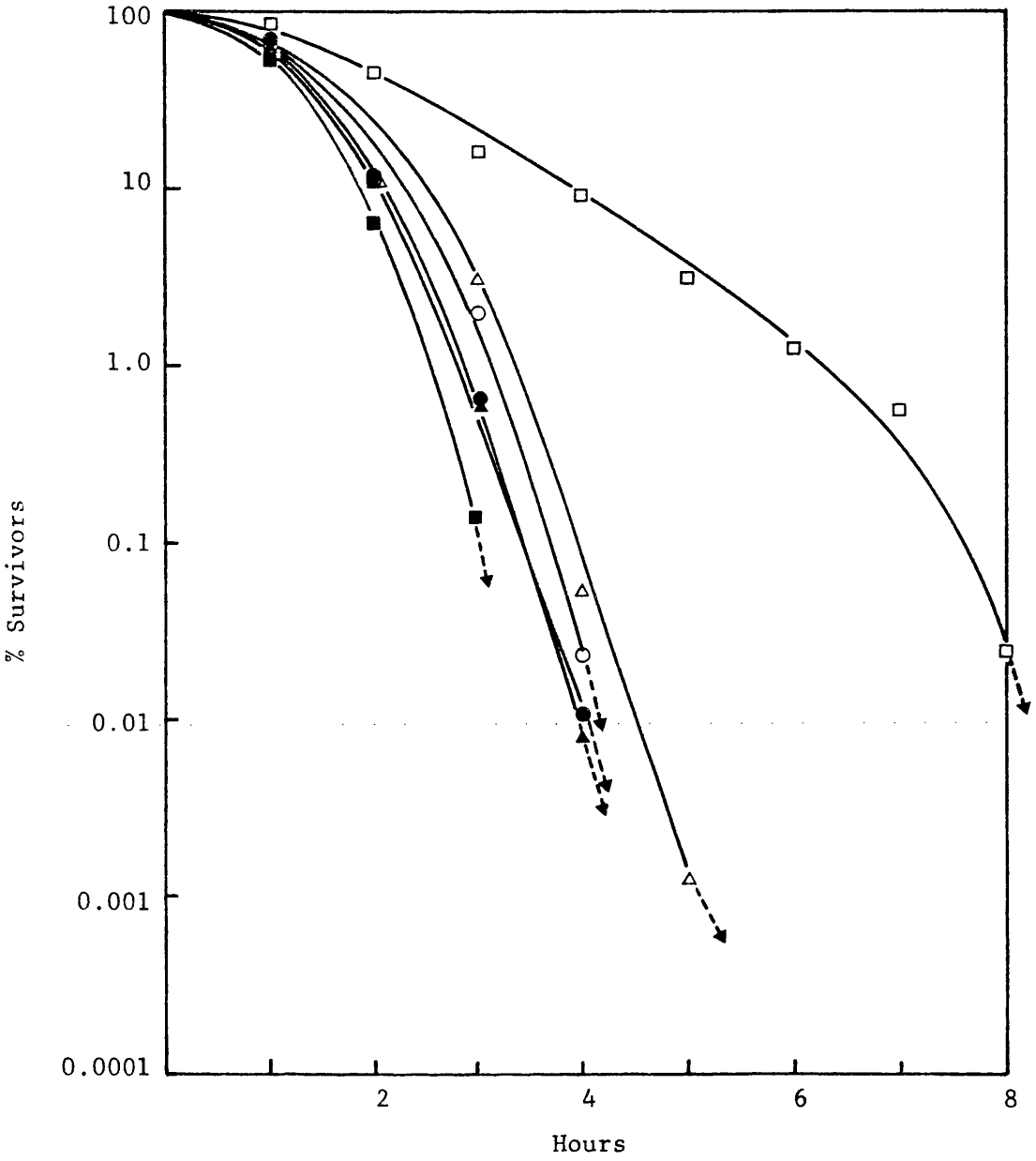


Fig.76 Effect of undegraded (□), 2-day (●), 4-day (▲), 6-day (■), 8-day (○) and 10-day (△) degraded solutions of thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0, with 0.1% w/v EDTA, at 25°C, on C. albicans.

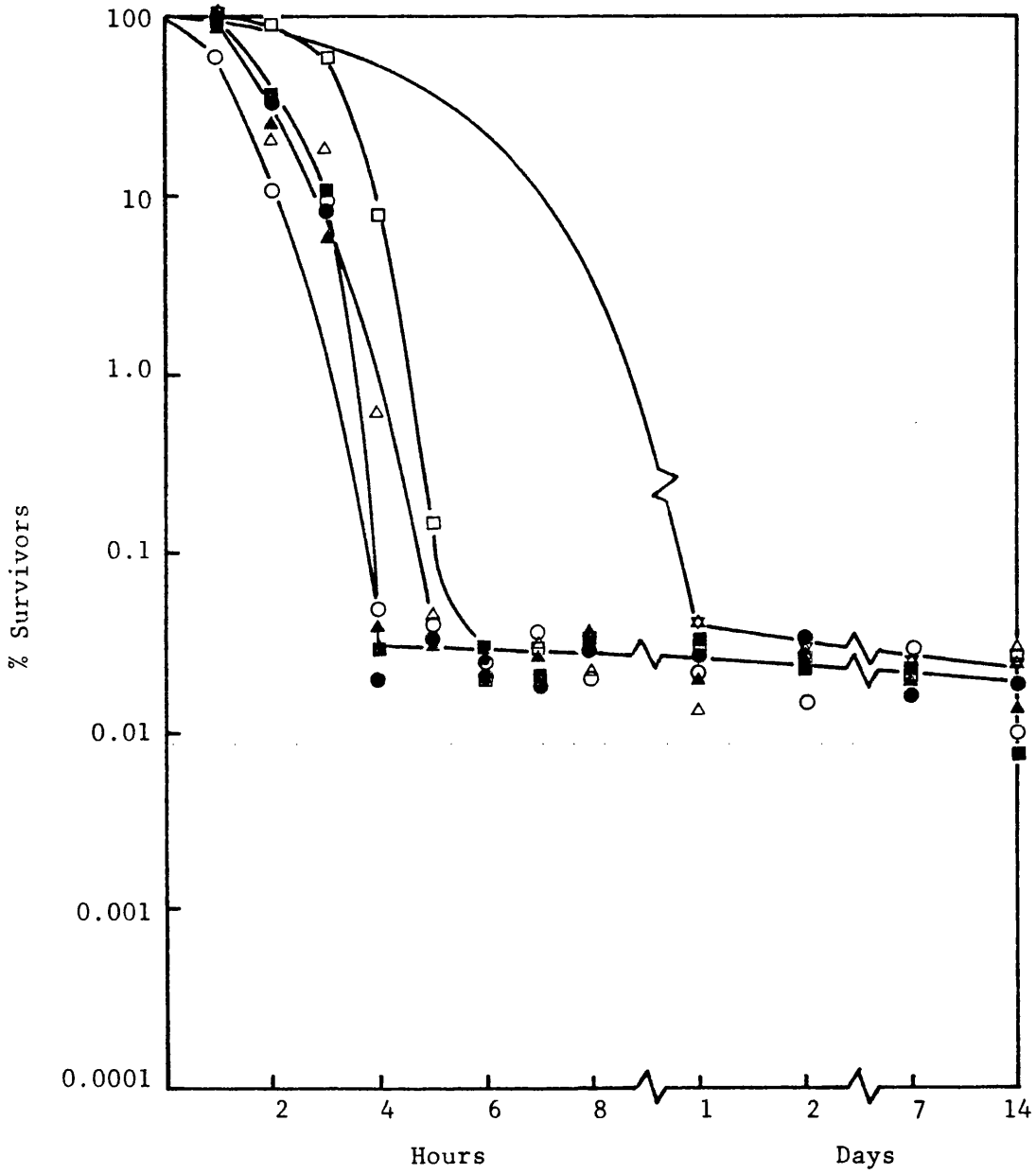


Fig.77 Effect of undegraded (□), 2-day (●), 4-day (▲), 6-day (■), 8-day (○) and 10-day (△) degraded solutions of thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0, with 0.1% w/v EDTA, at 25°C, on B. subtilis.

(✡) survival in buffer with 0.1% w/v EDTA

TABLE 35

‡ $t_{0.1}$ values recorded from the antimicrobial activity of photochemically degraded solutions of thiomersal in buffer with 0.1% w/v EDTA.

Time in Light Box (Days)	$t_{0.1}$ values (hours) ‡ Staph. aureus	Ps. aeruginosa	E. coli	C. albicans	B. subtilis
0	58.0	2.48	7.25	7.6	6.2
2	28.0	1.35	4.80	3.5	5.0
4	29.5	1.52	5-6	3.5	5.0
6	46.0	1.48	5.00	3-4	4.4
8	52.5	1.63	4.80	3.7	4.2
10	33.5	2.44	4.72	3.9	3.9

‡ t_{10} values recorded for Staph. aureus.

ANALYTICAL STUDIES

PRELIMINARY INVESTIGATIONS

Figure 78 is a chromatogram of freshly-prepared 0.008% w/v thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0 obtained using the HPLC analytical system developed. It can be seen that the system gives good resolution of the thiomersal peak; moreover, when EDTA was included in the formulation, no interference with the thiomersal peak was found. The retention time for thiomersal was shown to be 4 minutes 13 seconds, under the experimental condition used.

In order to ensure that all degradation occurring in thiomersal test solutions in the light box was as a result of photochemical changes in these, ampoules that had had light excluded from them whilst in the light box, were analysed by HPLC. Table 36 compares peak height measurements obtained from HPLC traces of freshly-prepared thiomersal with solutions that had been in the light box for 2-10 days in foil-wrapped ampoules; these were analysed statistically. It can be seen that there is no significant difference between these solutions, so that, when protected from light, no degradation occurred at the temperatures in the light box.

The effect of storing ampoules of thiomersal solution in the dark at $4^{o}C$ and at room temperature for ten days was also examined. The ampoules were analysed by HPLC and the results and a statistical analysis of the thiomersal peak heights obtained are recorded in Table 37. From this, it can be seen that when protected from light, no degradation of thiomersal occurred during either storage condition.

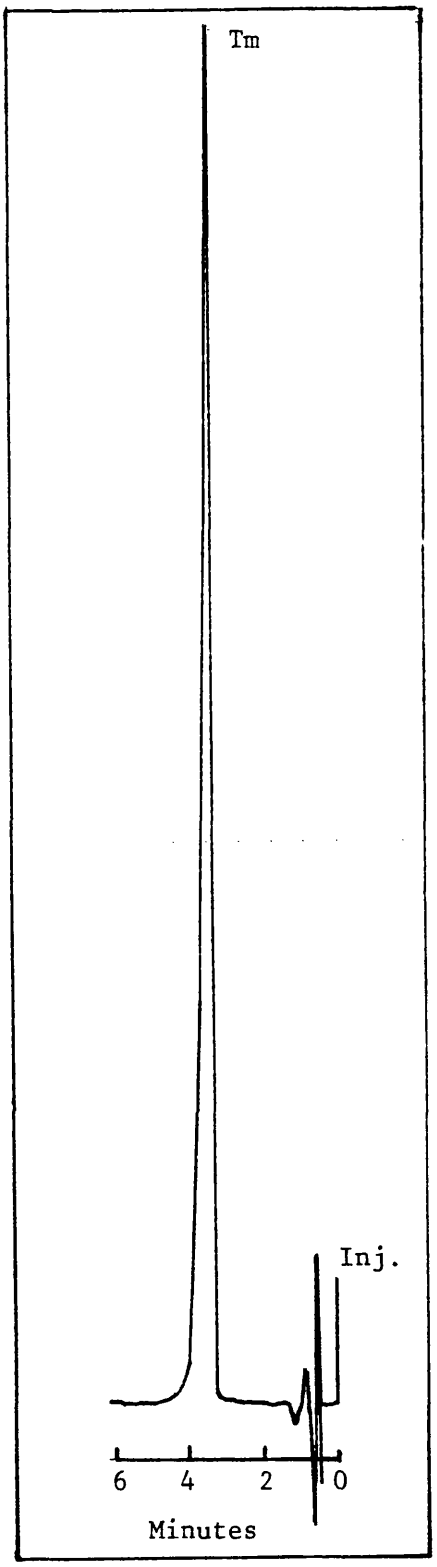


Fig.78 Chromatogram of 0.008% w/v thiomersal (Tm) in isotonic Sörensen's phosphate buffer, pH 7.0.

Column: 100 x 5mm, ODS-HYPERSIL
Mobile phase: Acetonitrile:Water:CTAB(0.01M):Sörensen's phosphate buffer, pH 5.8 (2/15M) 38:40:12:10
Flow rate: 2.2 ml per minute
Detector: uv at 235nm
Sensitivity: 0-0.04 AUFS
Load: 20 µl
Temperature: 30°C

TABLE 36a

Peak heights of thiomersal from HPLC chromatograms.

Test solutions prepared in isotonic Sörensen's phosphate buffer, pH 7.0, and stored in the light box for 2-10 days, protected from light.

Days in Light box	Peak Heights (mm)			Mean Peak height (mm)
0	168.5	168.0	171.0	169.17
2	174.0	169.0	168.0	170.33
4	171.5	166.5	165.5	167.83
6	172.0	164.5	169.0	168.50
8	171.0	167.0	169.0	169.00
10	167.5	170.0	169.5	169.00

TABLE 36b

Analysis of variance.

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Squares	Variance Ratio
Treatments	5	10.24	2.048	0.28
Residual	12	86.5	7.208	
Total	17			

$F_{0.05}$ (5,12) = 3.11 $F_{0.01}$ (5,12) = 5.06

Conclusion

The result is not significant at the 5% or 1% levels.

TABLE 37a

Peak heights of thiomersal from HPLC chromatograms.

Test solutions prepared in isotonic Sörensen's phosphate buffer, pH 7.0, and stored at $4^{o}C$ and at room temperature, protected from light.

Storage Conditions	Peak Heights (mm)				Mean Peak Height (mm)
Freshly-prepared	172.0	164.5	177.0	175.0	172.13
10 days at $4^{o}C$	171.5	170.0	173.5	167.5	170.63
10 days at room temperature	169.0	165.5	172.0	170.5	169.25

TABLE 37b

Analysis of Variance.

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Squares	Variance Ratio
Treatments	3	16.55	5.52	0.33
Residual	8	132.62	16.58	
Total	11			

$F_{0.05}\ (3,8) = 4.07$ $F_{0.01}\ (3,8) = 7.59$

Conclusion

The result is not significant at the 5% or 1% levels.

STUDIES ON PHOTOCHEMICALLY DEGRADED THIOMERSAL SOLUTIONS

Figures 79-83 are the HPLC traces obtained for thiomersal solutions degraded for 2-10 days in buffer, and in buffer with 0.01% and 0.1% w/v EDTA. Several observations may be made on the chromatograms obtained.

When degraded solutions had been prepared in buffer only, by Day 2, several peaks were apparent, both before and after the thiomersal peak. The peak heights of the degradation products can be seen to increase with an increased holding time in the light box, and are accompanied by a decrease in the height of the thiomersal peak. By Day 8, no detectable thiomersal remained and at Day 10, the peak height of the degraded products appeared to have stabilised, so that only minimal changes are apparent.

When prepared in buffer with 0.01% w/v EDTA, numerous peaks are detectable, both before and after the thiomersal peak, with a 2-day exposed solution; seven peaks were found to occur after the thiomersal peak. By Day 4, the thiomersal peak height was severely reduced and the peak heights of the products detected prior to thiomersal had increased; there was very little change in the peak heights of products occurring after thiomersal. By Day 6, no thiomersal could be detected, and even more degradation products are apparent on the trace. The large peak that occurred at about 10 minutes had degraded and was much reduced in height. At Days 8 and 10, although minor modifications in peak heights and shapes occurred, no fresh peaks were detectable; indeed, with time, the number of peaks found was reduced.

An effect immediately apparent with degraded solutions in buffer containing 0.1% w/v EDTA, is the increased height of the thiomersal peak

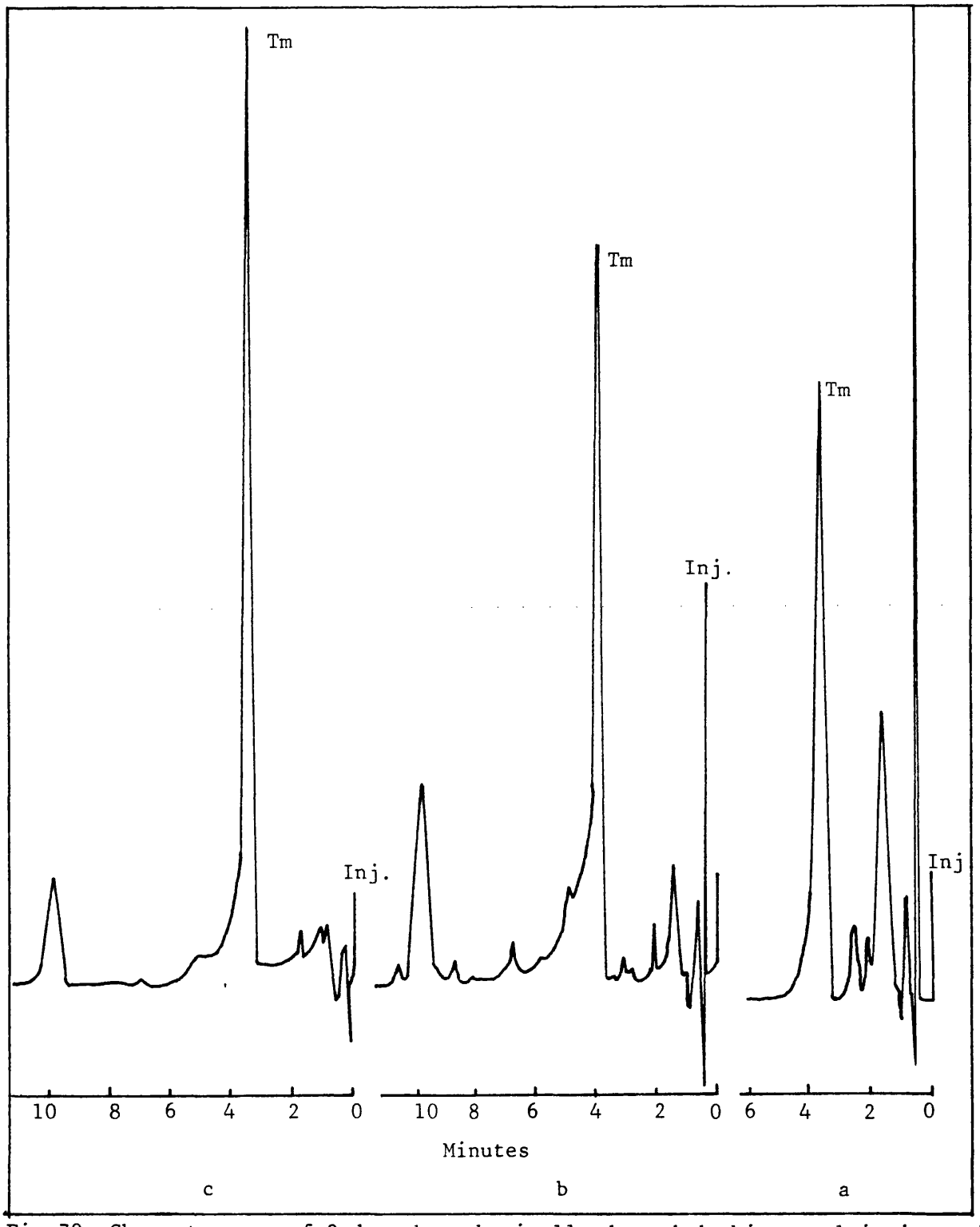


Fig.79 Chromatograms of 2-day photochemically degraded thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0, without EDTA (a), with 0.01% w/v EDTA (b) and with 0.1% w/v EDTA (c).

Column: 100 x 5mm, ODS-HYPERSIL
Mobile phase: Acetonitrile:Water:CTAB(0.01M):Sörensen's phosphate buffer, pH 5.8(2/15M) 38:40:12:10
Flow rate: 2.2ml per minute
Detector: uv at 235nm
Sensitivity: 0-0.04 AUFS (a, b); 0-0.08 AUFS (c)
Load: 20 µl
Temperature: 30°C

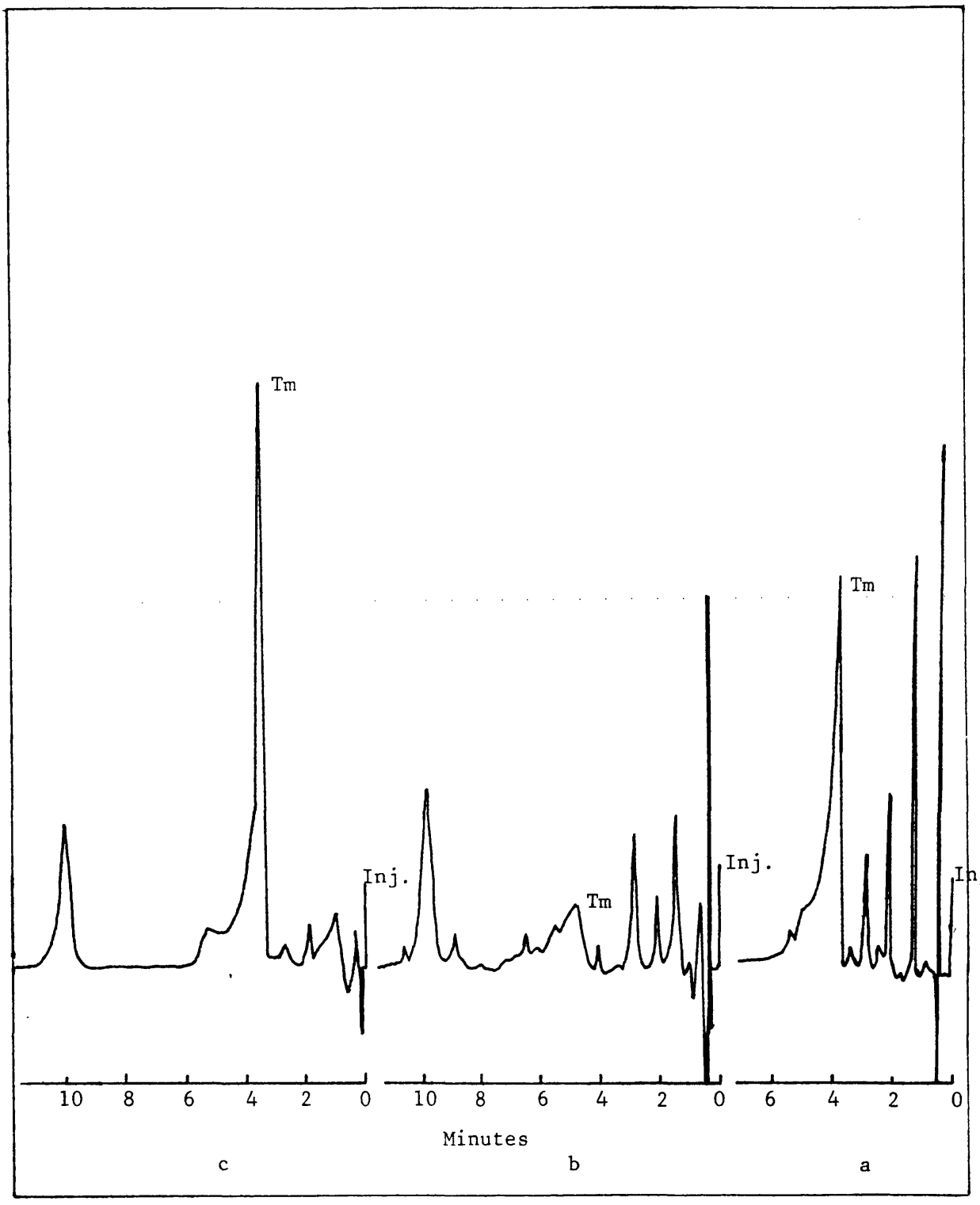


Fig.80 Chromatograms of 4-day photochemically degraded thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0, without EDTA (a), with 0.01% w/v EDTA (b) and with 0.1% w/v EDTA (c).

Column: 100 x 5mm, ODS-HYPERSIL
Mobile phase: Acetonitrile:Water:CTAB(0.01M):Sörensen's phosphate buffer, pH 5.8(2/15M) 38:40:12:10
Flow rate: 2.2ml per minute
Detector: uv at 235nm
Sensitivity: 0-0.04 AUFS (a, b); 0-0.08 AUFS (c)
Load: 20 μl
Temperature: 30°C

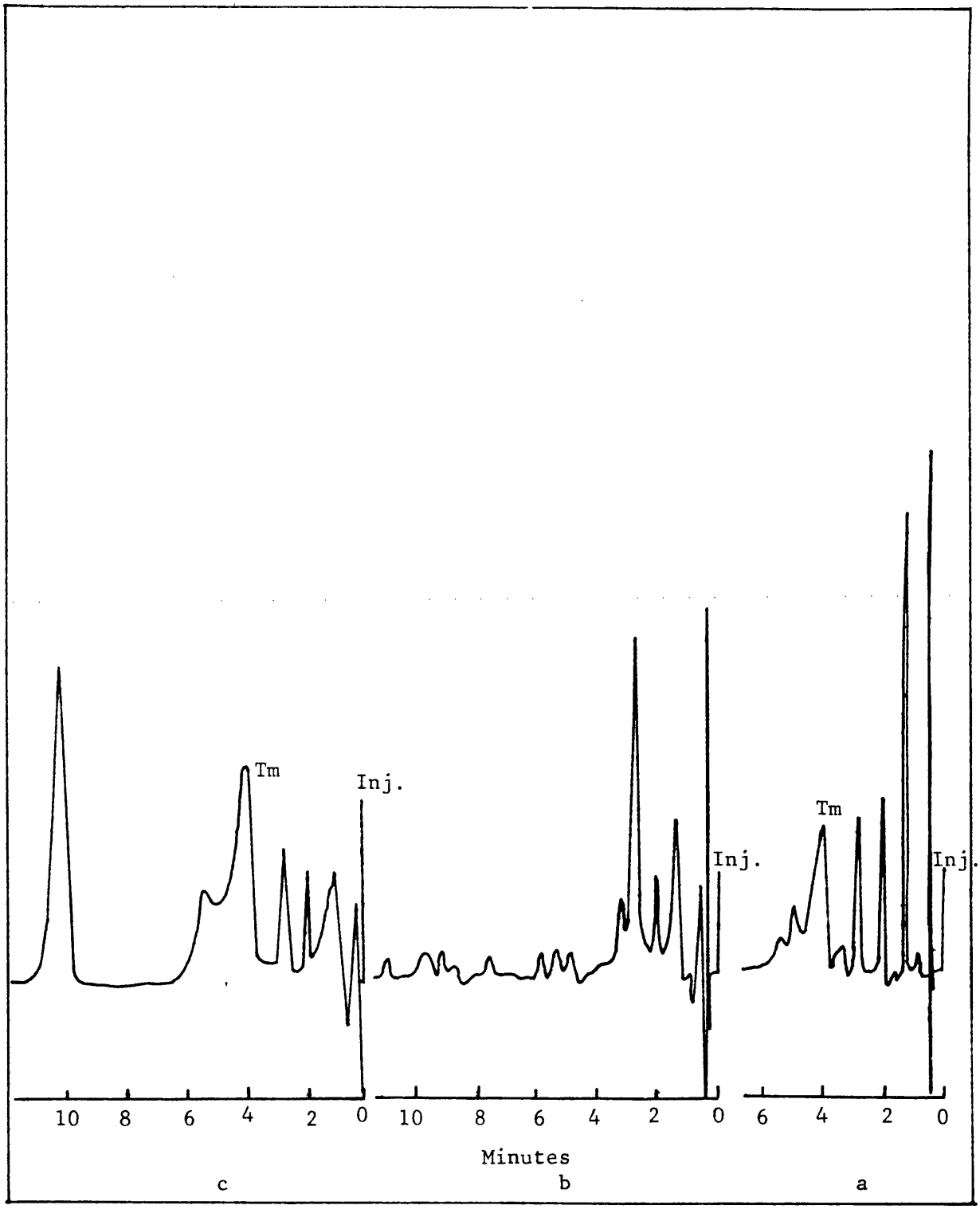


Fig.81 Chromatograms of 6-day photochemically degraded thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0, without EDTA (a), with 0.01% w/v EDTA (b) and with 0.1% w/v EDTA (c).

Column: 100 x 5mm, ODS-HYPERSIL
Mobile phase: Acetonitrile:Water:CTAB(0.01M):Sörensen's phosphate buffer, pH 5.8(2/15M) 38:40:12:10
Flow rate: 2.2ml per minute
Detector: uv at 235nm
Sensitivity: 0-0.04 AUFS
Load: 20 µl
Temperature: 30°C

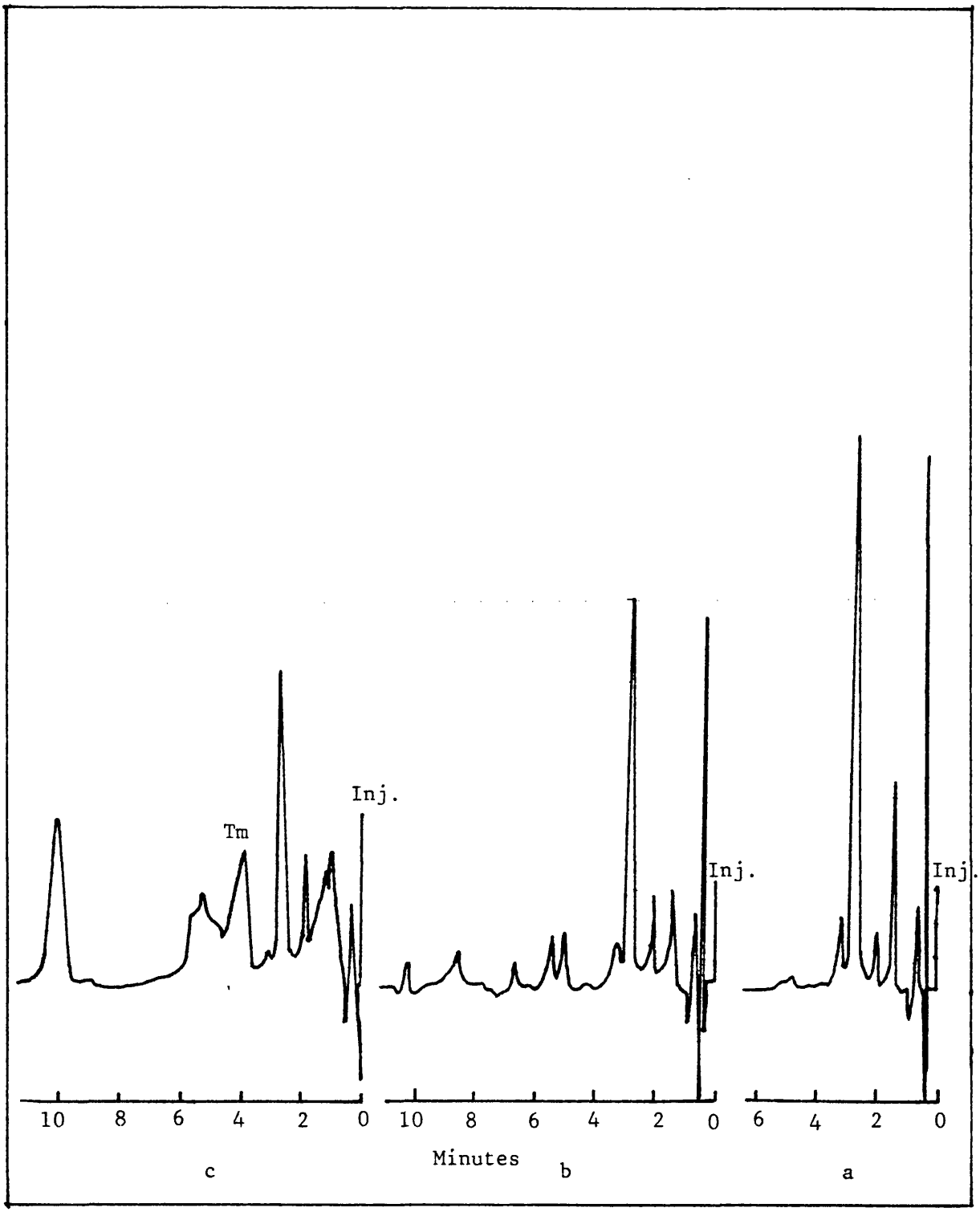


Fig.82 Chromatograms of 8-day photochemically degraded thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0, without EDTA (a), with 0.01% w/v EDTA (b) and with 0.1% w/v EDTA (c).

Column: 100 x 5mm, ODS-HYPERSIL
Mobile phase: Acetonitrile:Water:CTAB(0.01M):Sörensen's phosphate buffer, pH 5.8(2/15M) 38:40:12:10
Flow rate: 2.2ml per minute
Detector: uv at 235nm
Sensitivity: 0-0.04 AUFS
Load: 20 µl
Temperature: 30°C

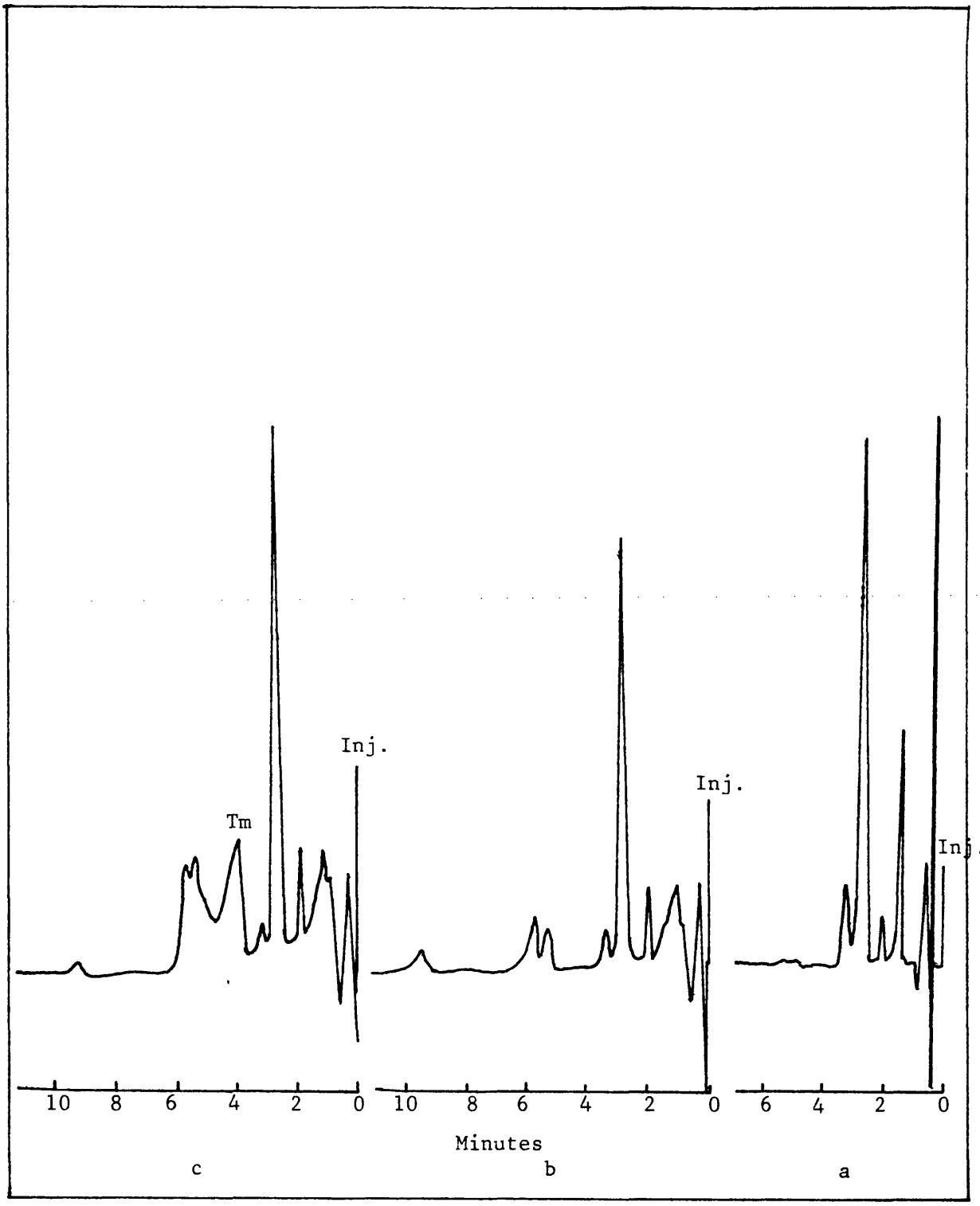


Fig.83 Chromatograms of 10-day photochemically degraded thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0, without EDTA (a), with 0.01% w/v EDTA (b) and with 0.1% w/v EDTA (c).

Column: 100 x 5mm, ODS-HYPERSIL
Mobile phase: Acetonitrile:Water:CTAB(0.01M):Sörensen's phosphate buffer, pH 5.8(2/15M) 38:40:12:10
Flow rate: 2.2ml per minute
Detector: uv at 235nm
Sensitivity: 0-0.04 AUFS
Load: 20 µl
Temperature: 30°C

on the Day 2 trace, when compared with that in the other two Day 2 chromatograms. The numbers and heights of other peaks were consequently much reduced. At Day 4, there was a reduction in the thiomersal peak height, and a slight increase in the height of the peak occurring at about 10 minutes; only small changes are seen in the rest of the peaks. By Day 6, the thiomersal peak was much reduced, but the heights of the other peaks had increased. No fresh peaks were detected though, and this contrasts sharply with the trace obtained for Day 6 solutions prepared in buffer with 0.01% w/v EDTA. At days 8 and 10, the thiomersal peak height was found to decrease slightly, but the most dramatic change was seen in the peak that occurs between 2-4 minutes; this has almost doubled in size at Day 10. At the same time, the peak that occurred at about 10 minutes had decreased greatly in size, so that very little of this product remained.

In the above chromatograms, confirmation of the retention time for thiomersal in degraded solutions was obtained by injecting a sample of the appropriate solution containing a suitable concentration of thiomersal immediately after the injection of a degraded solution. Thus, the exact position of thiomersal in a trace of a degraded solution could be established, despite minor fluctuations in mobile phase composition.

Figure 84 depicts the percentage residual thiomersal concentration found at each time interval tested.

To determine whether the observed antimicrobial activity of thiomersal could be related to a drop in its peak height, or indeed whether any degradation had occurred during the challenge experiments, a further series of HPLC analyses were undertaken. Immediately after the microbial

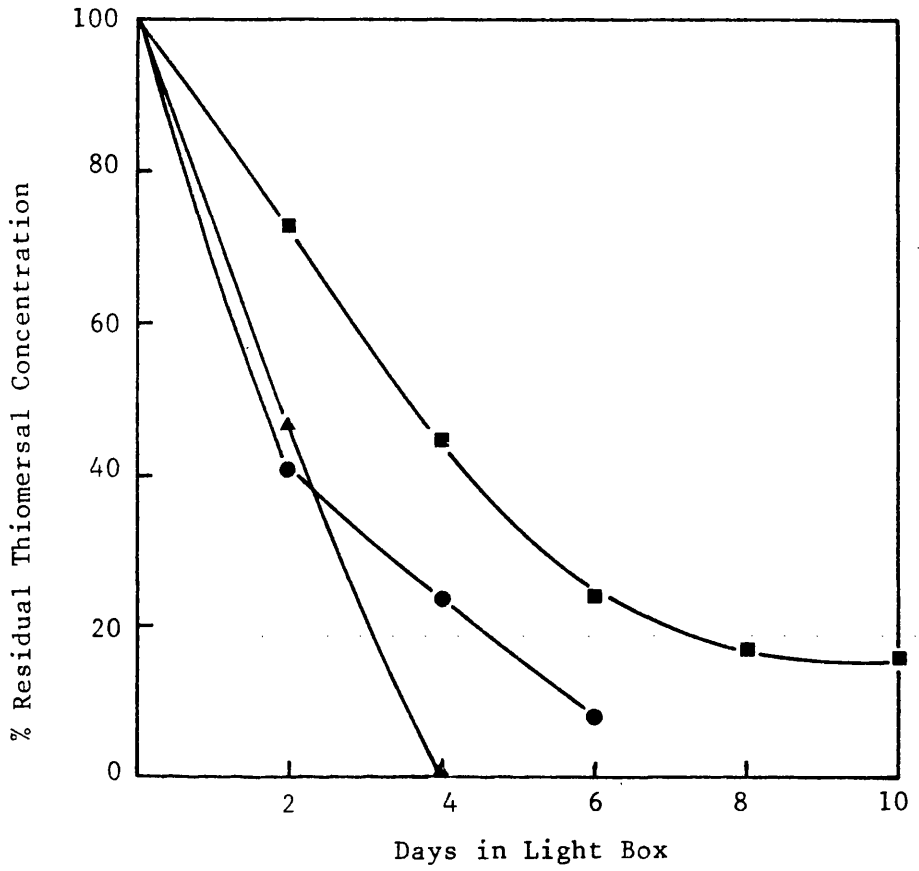


Fig.84 Percentage residual thiomersal concentration after photodegradation in isotonic Sörensen's phosphate buffer, pH 7.0, without EDTA (●), with 0.01% w/v EDTA (▲) and with 0.1% w/v EDTA (■). (Each point is the mean of data from two experiments).

challenge experiments, solutions were filtered through a 0.22μm membrane filter, to remove the organisms and stored in the dark at 4°C until they could be analysed. An identical thiomersal solution which had had no contact with any micro-organism, but had been held under the same experimental conditions, was used as a control. The chromatograms obtained from HPLC analyses showed that only a thiomersal peak was detected in both cases, i.e., that no degradation product was apparent. Table 38 is a statistical analysis of peak heights obtained for undegraded thiomersal solutions compared with those obtained where solutions had had eight hours contact with Ps. aeruginosa (10^6 cfu ml^{-1}) at 25°C. From this, it can be seen that although a drop in the thiomersal peak height was recorded after this bacterial contact, it was not found to be significantly different. This was also found to be true of data for the remaining test organisms.

When photochemically degraded thiomersal solutions were analysed by HPLC after microbial challenge experiments, however, differences in peaks and peak heights were immediately apparent. The chromatograms of 6-day degraded solutions of thiomersal in buffer, with and without EDTA, with no bacterial contact and after microbial challenge experiments with Ps. aeruginosa and Staph. aureus are presented in Figures 85-87. It can be seen that the peak heights of both thiomersal and its degradation products vary between the test organisms. The implications of this will be discussed later.

INACTIVATION STUDIES ON THIOMERSAL

To confirm that 0.01% w/v thiomersal, the highest concentration used in this study, was completely inactivated by the concentration of sodium thioglycollate determined to be necessary from bacterial

TABLE 38

Peak heights of undegraded thiomersal solutions from HPLC chromatograms of solutions with no bacterial contact and after microbial challenge experiments.

Test Organism: Ps. aeruginosa NCTC 6750

Experimental Condition (8h at 25°C)	Mean Peak Height (mm)	Student 't' test (n=10) Tab	Cal
No bacterial contact	169.0	2.75	0.78
After bacterial contact	167.0		

Conclusion

The results are not significant at the 5% level.

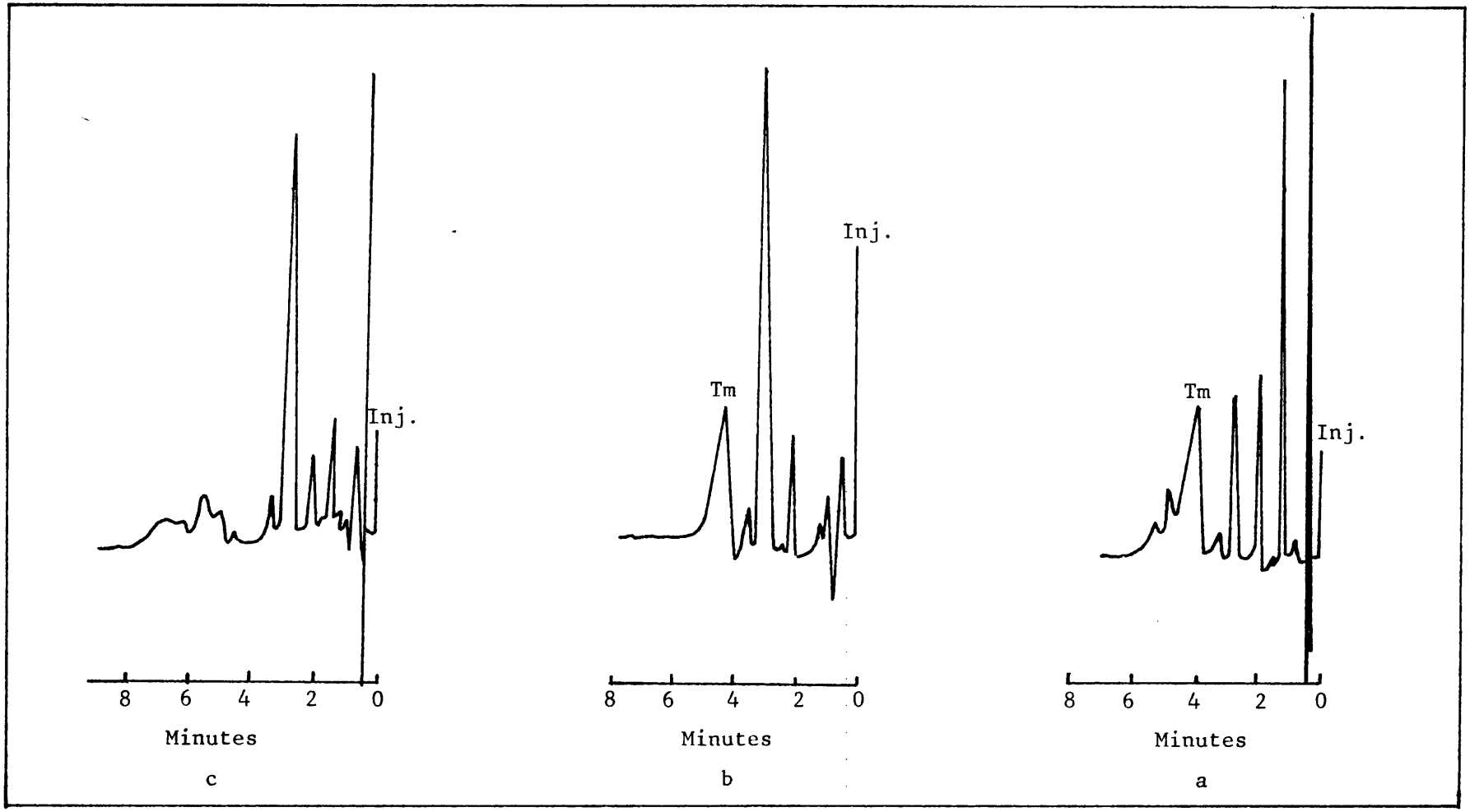


Fig.85 Chromatograms of 6-day photochemically degraded thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0, with no bacterial contact (a), after 80h contact with Staph. aureus (b) and after 8h contact with Ps. aeruginosa (c).

Column: 100 x 5mm, ODS-HYPERSIL
Mobile phase: Acetonitrile:Water:CTAB(0.01M):Sörensen's phosphate buffer, pH 5.8(2/15M) 38:40:12:10
Flow rate: 2.2ml per minute Detector: uv at 235nm
Sensitivity: 0-0.04 AUFS Load: 20 µl
Temperature: 30°C

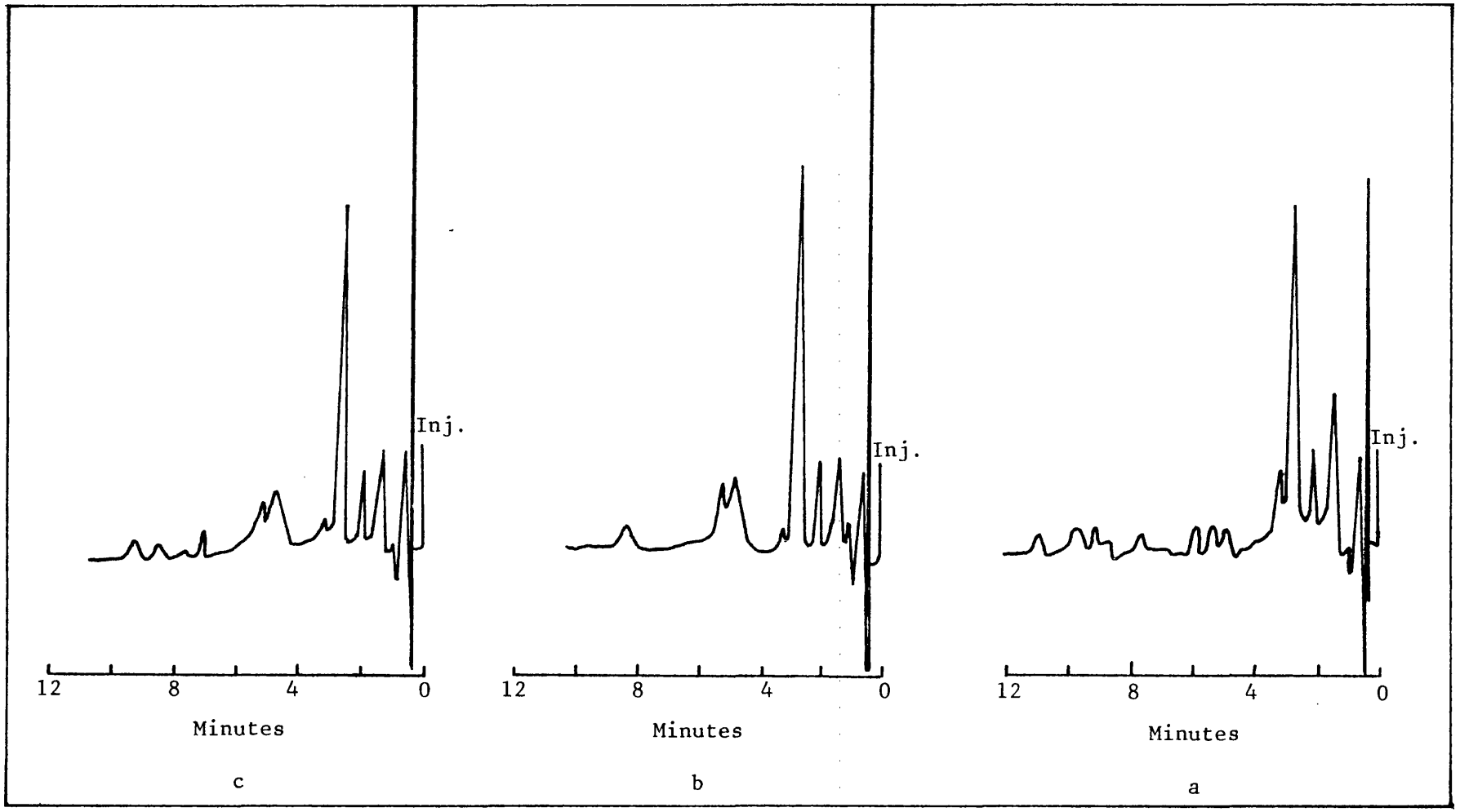


Fig.86 Chromatograms of 6-day photochemically degraded thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0, with 0.01% w/v EDTA, with no bacterial contact (a), after 80h contact with Staph. aureus (b) and after 8h contact with Ps. aeruginosa (c).

Mobile phase: Acetonitrile:Water:CTAB(0.01M):Sörensen's phosphate buffer, pH 5.8(2/15M) 38:40:12:10
Column: 100 x 5mm, ODS-HYPERSIL
Sensitivity: 0-0.04 AUFS
Load: 20 µl
Flow rate: 2.2ml per minute
Detector: uv at 235nm
Temperature: 30°C

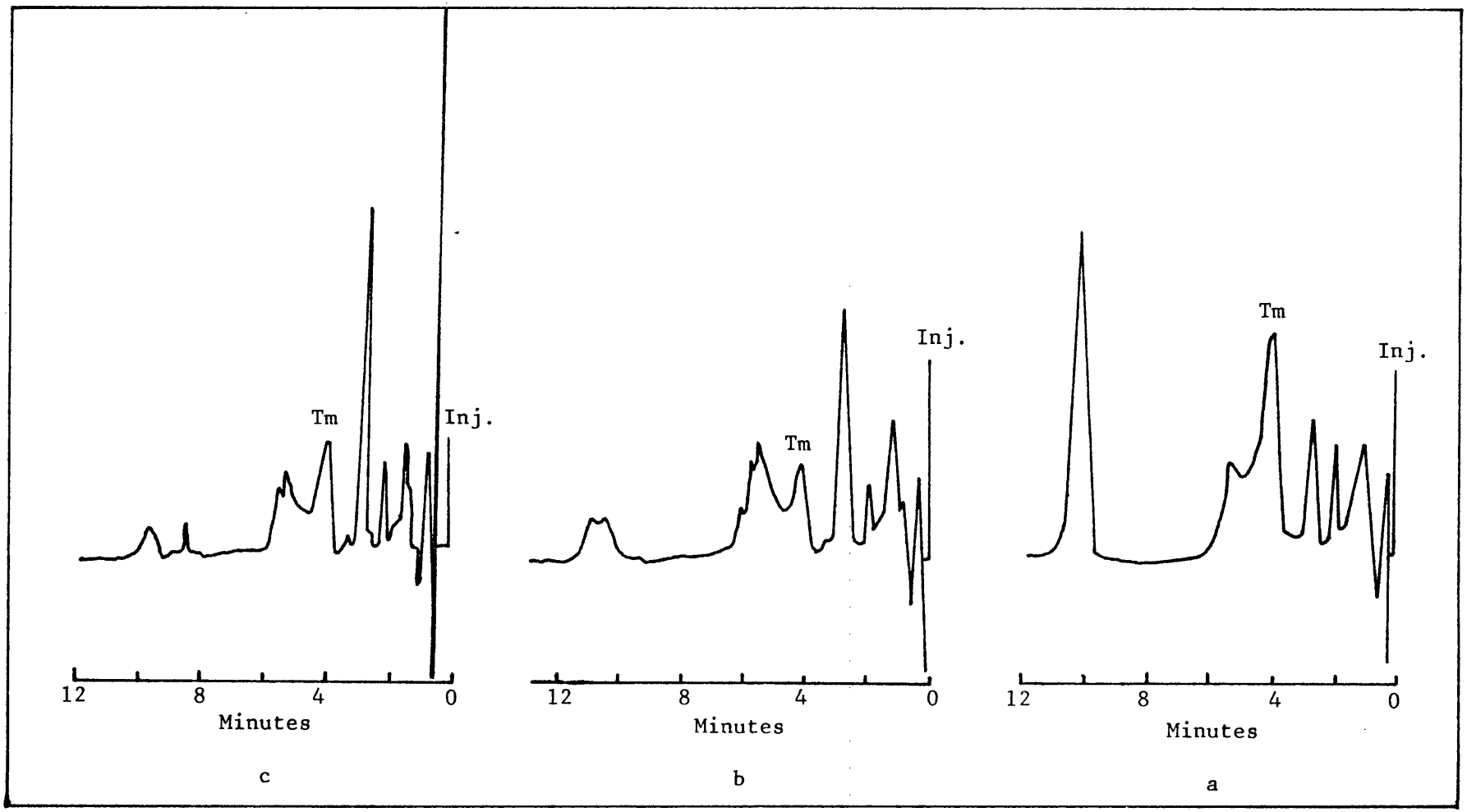


Fig.87 Chromatograms of 6-day photochemically degraded thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0, with 0.1% w/v EDTA, with no bacterial contact (a), after 80h contact with Staph. aureus (b) and after 8h contact with Ps. aeruginosa (c).

Mobile phase: Acetonitrile:Water:CTAB(0.01M):Sörensen's phosphate buffer, pH 5.8(2/15M) 38:40:12:10
Column: 100 x 5mm, ODS-HYPERSIL
Sensitivity: 0-0.04 AUFS
Load: 20 µl
Flow rate: 2.2ml per minute
Detector: uv at 235nm
Temperature: 30°C

experiments (Appendix II, page 279), a further series of experiments were carried out. This involved diluting the thiomersal in 1% sodium thioglycollate, prepared here in water, as it would be in a challenge experiment. Immediately after, the test solution was filtered through a 0.2 μm membrane filter and injected onto the analytical column. Figure 88 is a chromatogram of 1.0% w/v sodium thioglycollate in distilled water and that obtained after the addition of 0.01% w/v thiomersal in an appropriate dilution. It can be seen that no thiomersal appears on the trace and that, at about 17 minutes, a peak appeared on all traces where thiomersal had been added to sodium thioglycollate solutions, but not when sodium thioglycollate or thiomersal were injected on their own.

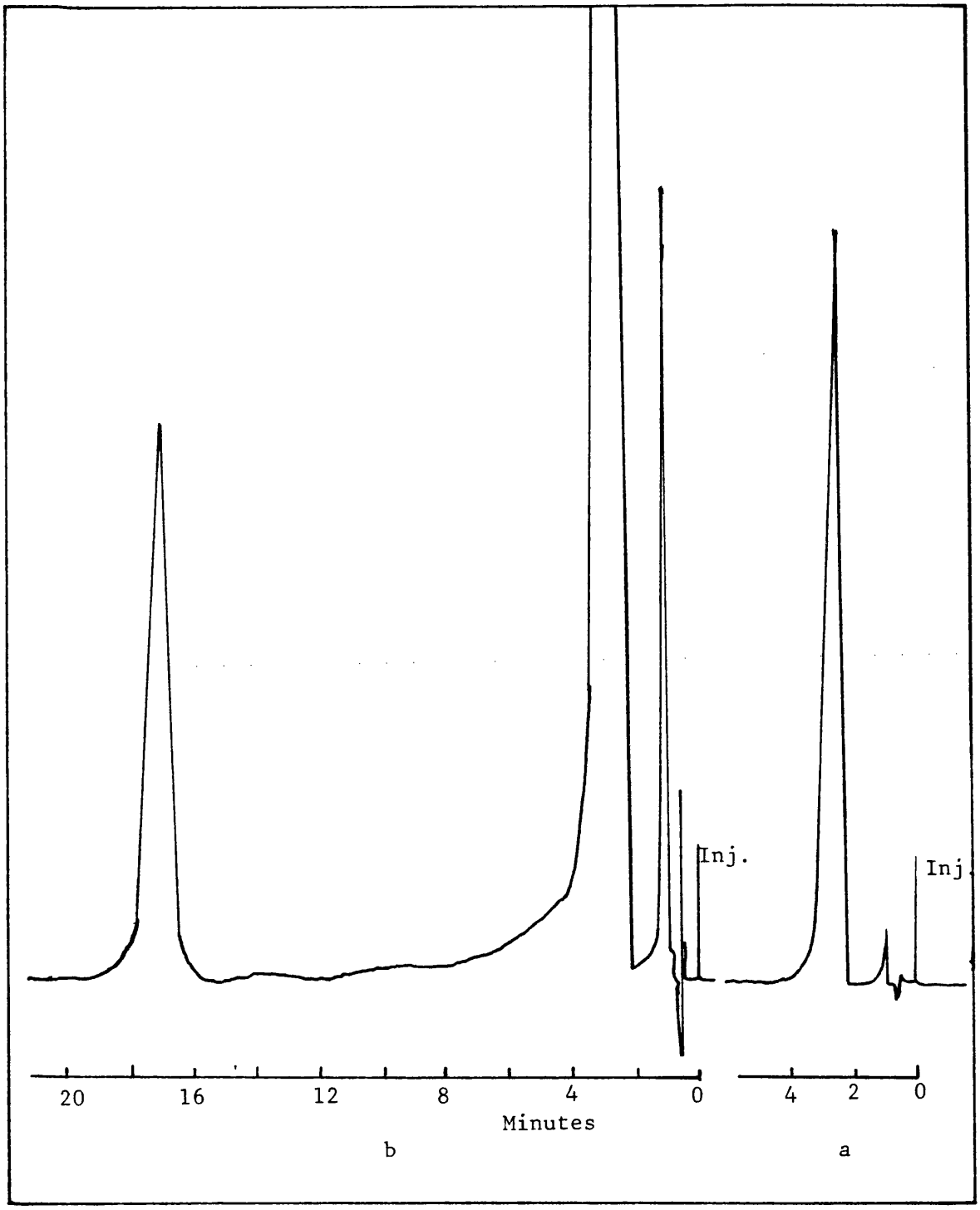


Fig.88 Chromatograms of 1.0% w/v sodium thioglycollate in distilled water, before and after addition of 0.01% w/v thiomersal in distilled water (a & b respectively).

Column: 100 x 5mm, ODS-HYPERSIL
Mobile phase: Acetonitrile:Water:CTAB(0.01M):Sörensen's phosphate buffer, pH 5.8(2/15M) 38:40:12:10
Flow rate: 2.2ml per minute
Detector: uv at 235nm
Sensitivity: 0-0.64 AUFS (a); 0-0.04 AUFS (b)
Load: 20 µl
Temperature: 30°C

DISCUSSION

DISCUSSION

The initial phase of this work was concerned with the development of a suitable chemically-defined medium for the growth of the selected test organisms. It was felt that the use of a defined medium would increase test reproducibility and eliminate the possibility of batch-to-batch variations, known to occur with commercial media. It had been intended originally to use the growth medium developed for Staph. aureus for all the test organisms; however, preliminary growth studies with Ps. aeruginosa and C. albicans showed that this was not feasible. With Ps. aeruginosa, excessive amounts of slime were produced when this organism was grown in the basal medium supplemented with vitamins and 10 amino acids. As the filter rapidly became clogged, this made harvesting of the organism by membrane filtration difficult; re-suspending the organisms from the filter was also a problem, as the slime caused the organisms to stick to the filter despite extensive whirlimixing. As this organism is known to have simple nutritional requirements, it should have been able to grow in the unsupplemented basal medium. It was found, however, that inoculating this directly from a TSA slope, resulted in a long lag phase during which growth, when it occurred, took the form of tiny aggregates. This was not considered satisfactory. When the basal medium was inoculated from a culture grown initially on a basal medium + agar plate, however, although some aggregates were observed in the early stages of growth, turbidity was consistently found after 24h at 37°C. Transferring an organism from an entirely organic medium to a wholly inorganic medium involves it having to synthesise a range of different enzymes before successful growth can commence. This is the normal reason for the long lag periods observed when such transfers occur. Growing the Ps. aeruginosa on the basal medium + agar prior to inoculating it into the liquid basal medium, enabled the organism to

achieve this initial transition whilst growing as colonies, where possibly the impurities known to occur in agar facilitated such a change. Once these new enzymes had been synthesised, growth in the liquid basal medium could proceed smoothly, as indeed was shown to be the case.

With C. albicans, an extended lag phase of more than 24 hours was obtained when this organism was inoculated from a TSA slope into the basal medium supplemented with the ten amino acids and vitamins. When a Casamino acid supplement (vitamin-free), was included in the medium, however, turbidity was observed within 24 hours at 37°C. Apparently, C. albicans has a specific requirement for some amino acid not present in the orginal ten. This increased need for a nitrogen source as compared with Staph. aureus, may be appreciated when one considers the normal habitat of these two potentially pathogenic micro-organisms. Both are human commensals, with Staph. aureus being able to colonise 30-40% of certain restricted sites on the skin, especially the anterior nares of healthy persons; C. albicans is found to occur in the mouth and faeces of 20-30% of healthy persons (184). Thus, this yeast usually has access to a much richer supply of organic matter and has been able to maintain its fastidious requirements. This reason does not account, however, for the successful growth of E. coli in basal medium only when inoculated from a TSA slope, as E. coli strains predominate among the aerobic commensal organisms, present in the healthy gut. E. coli has beeen suggested, however, as an example of an organism with highly efficient synthetic powers (192), and this may account for its smooth transition from a totally organic to an entirely inorganic medium. B. subtilis was found to grow successfully in the medium developed for Staph. aureus.

Figures 11-15 (pages 103-107), illustrate the growth of the test organisms in their respective defined media. Growth in TSB was followed for Staph. aureus and Ps. aeruginosa to determine how growth in the chemically defined medium developed, compared with that in a standard organic medium. The results show that whilst an anticipated increase in viable count was obtained when grown in TSB, the shapes of the curves for both media types were essentially the same, although levelling-off in the stationary phase occurred much later in the defined medium. An interesting feature of both curves obtained for Staph. aureus, is the presence of a small plateau region at 8-12 hours. This type of bi-phasic growth curve is normally obtained when an organism is grown in a medium which has two carbon sources; the preferred carbon source is used first and the plateau region represents the period during which a further set of enzymes is synthesised to utilise the second source. It seems unlikely that this has happened here. Staph. aureus is a facultative anaerobe as well as an aerobe, and if conditions in the growth medium were such that oxygen levels were low, the organism may have switched to reducing pyruvate to lactate and ethanol, rather than oxidising it to carbon dioxide and water. This would involve synthesising a different set of enzymes and could explain the period of slow growth that occurred in both media. Gale (192) suggested that the staphylococci represent advanced organisms as their fermentation of glucose approached a lactic one. The nature and relative proportions of the products were apparently influenced markedly by growth conditions and pH of the medium.

To eliminate the possibility of any organic medium being carried over which might affect their behaviour, test organisms were only harvested for challenge testing from secondary cultures. Moreover, as

micro-organisms are known to be able to accumulate 'nutrient pools' and endogenous reserves for several generations (193, 194), the possibility of these still being present in a secondary culture would be extremely low. As Figures 11-15 demonstrate, the lag phase is also much reduced in the second transfer, as the organisms are now adapted to the new medium. Thus, lag phases were under an hour for E. coli and B. subtilis and about two hours for the other test organisms.

At 22 hours, the time chosen to represent late stationary phase, the viable count was found to be about 1×10^9, 3×10^9, 2×10^9, 6×10^7 and 3×10^9 cfu ml^{-1}, for Staph. aureus, Ps. aeruginosa, E. coli, C. albicans and B. subtilis respectively. Total counts made at this time showed that about 90% of the organisms were viable. Cessation of growth or death of the organisms was initially thought to occur because of depletion of the carbon source or some other media component and/or an accumulation of toxic by-products. Studies were, therefore, carried out using Staph. aureus and Ps. aeruginosa, to investigate this. The micro-organisms were separated from their secondary culture medium after 22 hours growth, by centrifugation; they were washed twice and re-suspended in fresh growth medium and in growth medium with double glucose concentration. Growth was followed by viable counting and for both organisms in both media types 24 hours later, only a marginal increase in cfu ml^{-1} was obtained. This suggests that growth stopped not because of a shortage of glucose or some media component nor because of toxic by-products accumulating in the medium. Two other possible reasons for the cessation of growth are that a shortage of oxygen might restrict growth or that the pH of the medium might have fallen to a value which was unfavourable for growth as a result of the formation of the acid products of metabolism. The former was not checked, but is unlikely to be critical because both

test organisms are facultative anaerobes; when the pH of the media was measured before centrifugation, it was found to be about pH 6.9, and this is within the range tolerated by these bacteria.

Membrane filtration was selected as the method for harvesting the test organisms, because it is a quick and simple method of separating the cells from their growth medium. It was not considered desirable merely to use a dilution of the secondary growth culture, as the cells might be able to carry on growing slowly, and also because of the unknown contribution made by any waste by-products present. By carrying out all washing operations and re-suspending the organisms in the basal medium minus the carbon source, it was hoped that any traumatic effect normally associated with these manipulations would be kept to a minimum.

The concentration of test organisms ml^{-1} was determined from calibration plots that had been prepared using 22 hour secondary cultures of each organism. Optical density readings of suspensions of the test organisms were made at 470nm for all organisms apart from Ps. aeruginosa; this was read at 600nm because this organism produces a blue-green pigment, pyocyanin, which might interfere with readings at lower wavelengths. An examination of the literature reveals, however, that the wavelengths at which optical density readings are made vary tremendously. Determinations have been made at 420nm (44, 59, 195), and 540nm (180); some authors do not use a higher wavelength for Ps. aeruginosa (44, 59, 195). This may not be important when this organism is grown in complex commercial media, where pigmentation has been observed to vary and is often not visible when the organism is grown at 37°C; when grown in the defined medium developed here, however, a deep green colour was produced so that the use of a high wavelength, such as 600nm, does seem necessary

here. For the other test organisms, however, it would appear that the other wavelengths, such as 470nm or 540nm, could equally well have been used. The important criterion to be considered is that the calibration plots must always be prepared under exactly the same conditions as the test suspension. This is important, not only because the size of cells has been shown to vary with the stage of growth (147) and their light scattering properties would consequently be affected, but also because the proportion of living and dead organisms will also vary with the stage of growth, and measurements of turbidity cannot distinguish between these.

It had been decided that the antimicrobial effectiveness of thiomersal would be followed from plate counts, as although tube tests are convenient and have a place in the evaluation of disinfectants and preservatives, they do not provide any information on the type of kill obtained. Data generated from plate counts can be plotted semi-logarithmically to illustrate the destruction of the micro-organisms in relation to time. Several theories and models of microbial death have been proposed to take account of the effect of destruction by various agents (5, 196). An examination of the survivor curves obtained after treating the test organisms with different concentrations of thiomersal reveals that there is no standard shaped curve (Figures 16-20, pages 109-113). Although all curves are characterised by an initial 'shoulder' whose duration varied with the test organism as well as the concentration of thiomersal used, thereafter, any one of a number of possible shapes was found. These ranged from curves, both convex and concave in shape, to curves with an extended tail portion; more rarely, a straight line was obtained. Simple mathematical analysis of such complex curves is not posssible. Nevertheless, it was considered important to select some

criterion by which the results could be compared. In its recommendations for tests to determine the efficacy of antimicrobial preservatives in ophthalmic preparations, the BP 1980 states that the numbers of bacteria recovered per ml should be reduced by a factor of not less than 10^3 within 6 hours, and that no organisms should be recovered from 1ml at 24 hours and thereafter; the numbers of yeasts and moulds per ml were required to be reduced by a factor of not less than 10^2 within seven days of challenge and not to increase thereafter. It was decided, therefore, to base this criterion on the recommendation of the BP for bacteria and hence the $t_{0.1}$ values, i.e. the time taken to reduce the viable count by three log cycles, was selected. Where it was not possible to read $t_{0.1}$ values because of the extremely slow or quick death of the test organisms concerned, then $t_{1.0}$ or t_{10} values were read as alternatives. Whilst such a measure does not provide any information on the type of survivor curve obtained, it does enable the effectiveness of solutions to be related with changes in experimental conditions and between test organisms. A thiomersal solution was, therefore, said to be 'effective' if it succeeded in reducing the viable count by three log cycles within 6 hours.

Preliminary experiments carried out with thiomersal were concerned with determining the effect of concentration on its antimicrobial activity. Mercurials are reported to have low concentration exponents (197), so that it was anticipated that concentration would only have a marginal effect on activity; this was indeed shown to be the case (Figures 16-20, pages 109-113). An average concentration exponent of about 0.4 was found (Table 20, page 115), which is close to the reported value of 0.5 for organomercurials (198). In calculating the value of n for each test organism in this work, the times used were not that of

a total kill, but rather the $t_{0.1}$ values. It has been argued that the equation for n only fitted the observed facts when t was the actual kill time, and that if 90% or 99.9% mortality times were used, then different values of n would be obtained (199). The reason for this is probably that when the shoulder and the slope both change, a measure of each should ideally be obtained. The calculated value of n, however, does give an approximate measure of the effect of a change in concentration on the antimicrobial activity of a preservative and is useful for this purpose. Table 20 (page 115) also shows that n varies with a test organism, as has been found previously (200). The data in this Table were obtained by calculating concentration exponents using the two extreme concentration tested, namely, 0.001% and 0.01% w/v thiomersal. If instead, the 0.004% and 0.008% w/v concentrations are used, although the individual values vary between the test organisms, an average value of n of about 0.4 would be obtained, which is consistent with that previously calculated.

A serious outcome of the results of the concentration experiments is that none of the concentrations of thiomersal tested were 'effective' against Staph. aureus or C. albicans. 0.004%, 0.008% and 0.01% w/v thiomersal were 'effective' against both Ps. aeruginosa and B. subtilis, but only at 0.01% w/v was it 'effective' against E. coli. As thiomersal is normally used in contact lens solutions at concentrations of between 0.001-0.004% w/v, and in eye-drops at 0.01% w/v (27), the implications of these findings can readily be appreciated.

Some evidence that the variety of slopes obtained in the survivor curves is not an experimental artifact, is provided by the experiments illustrated in Figures 21-25 (pages 117-121). These show that the

curves were quite reproducible, apart from during the later stages of an experiment where colony numbers were very low and the experimental error consequently high. This was especially noticeable with E. coli for reasons which are not clear. Consequently, there were no surviving organisms at 96 hours for Staph. aureus, 8 hours for Ps. aeruginosa, and 9 hours for E. coli and C. albicans. With B. subtilis, however, a sigmoidal curve was obtained with a prolonged 'tail' portion that extended over the two week sampling period. These survivors were subsequently established as being the spore-bearing fraction of the inital inoculum, by a heat survival experiment (10 minutes at 80°C). These spores would be unaffected by the thiomersal and would, therefore, grow up as 'survivors' when sampled from the challenge mixture. It has been suggested (201), that metal cations such as mercury, chromium, copper or iron, are able to inhibit the germination of spores of bacilli. Mercury is reported to have a high degree of affinity for the spore surface and may inhibit the post-germination period, even after thorough washing (202). This may account for the occasional 'death' that occurred in some B. subtilis curves, where no survivors were detected.

All kill curves in this study are characterised by an inital 'shoulder' period, for which several explanations are possible. It can be seen that the shoulder is longest with Staph. aureus, and this could be partly due to the tendency of this organism to occur in 'grape-like' clusters. Access of the thiomersal to cells within a cluster would, therefore, be slow, and this could contribute to the long shoulder. Death of a colony forming unit would also only occur when all the cells in a cluster had died, and this would obviously take time. Another possible reason for the presence of the shoulder, is that this

may represent a period during which degradation of thiomersal occurs to give an active moiety, which then penetrates the cell and exerts an effect. This could happen in one of the following two ways.

1) Degradation of the thiomersal occurs, as in the equilibrium reaction proposed by Davisson et al (74), to give an active moiety, for example the $C_2H_5Hg^+$ ion, which is then responsible for the antimicrobial activity. The shoulder-period could then be a time during which this occurred, to build up an effective concentration of the $C_2H_5Hg^+$ ion.

2) The cell could synthesise an exo-enzyme which then attacks the thiomersal and produces an active antimicrobial moiety in the process. Certain micro-organisms have been shown to be able to de-toxify mercurial compounds metabolically, by forming volatile metallic mercury which is then eliminated from the culture medium as mercury vapour (203-205). These bacteria all carried the genes for determining mercury resistance on plasmids, which the test organisms here do not possess. It is unlikely though, that an exo-enzyme synthesis and release occurs during the shoulder period, as large amounts of enzyme would be needed to have an effect that would be of no benefit to the cell.

Finally, the shoulder may be related to the time needed to penetrate the cells of the test organisms, where differences in cell wall structure would be a contributory factor. A possible explanation for the shoulder period was derived from observations made on the colonial morphology of surviving organisms. These have been summarised in Table 22 (page 123), and it is interesting that with the three Gram-positive organisms tested an effect resulting in a decrease in colony size for some of the survivors was seen. These colonies remained small, despite continued incubation at 37°C, but with an increased contact time with thiomersal, they disappeared altogether. A possible

explanation for the formation of these small colonies may lie in the differences in cell wall composition of Gram-positive and Gram-negative organisms (Table 39). All bacterial cell walls contain mucopeptide which confers rigidity on the wall and enables it to withstand high internal osmotic pressures. The internal osmotic pressure of Staph. aureus cells has been found to be between 20-25 atmospheres (206), whereas that of typical rod-shaped organisms has been shown to be about 5-6 atmospheres (192). Mucopeptide forms about 65% of the wall of Staph. aureus, but less than 10% of the wall of E. coli (192). The picture that emerges from these findings is that the cell wall of a typical Gram-positive organism is much tougher than that of a Gram-negative organism. It was, therefore, anticipated that it would take longer for thiomersal to penetrate Gram-positive cells than Gram-negative cells. The atypical small colonies observed could represent survivors from, perhaps, the more sensitive members of a population, where some thiomersal has penetrated, sufficient to cause an effect seen as a small reduction in colony size, but insufficient to cause death. When sufficient thiomersal was present inside the cells to cause death, the effect disappeared. The effect was certainly not due to inadequate recovery media or to insufficient holding time in the recovery media because these had been tested and found to be satisfactory (pages 70-74). The possibility that the plating medium, TSA, might be responsible was thought to be unlikely, but nevertheless, two other commercially available media were compared for their general ability to recover survivors from the challenge experiments. The results of these experiments are illustrated in Figures 26-30 (pages 127-131). It can be seen that there is little difference between the recoveries obtained in these media types, apart from with C. albicans, where recovery on CAB was somewhat low, and with B. subtilis, where

TABLE 39

Some characteristic features of cell walls of typical Gram-positive and Gram-negative micro-organisms.

Cell Wall Components	Gram-positive	Gram-negative
Mucopeptide	50-80%	1-5%
Lipid	Little or none	High lipid content (10-20% of dry weight)
Amino acids	Limited range of amino acids	Full range of amino acids found in protein
Amino sugar content	High amino sugar content (15-20% of dry weight)	Low amino sugar content (2-5% of dry weight)

recovery on TSA was better than that on CAB or NA. Table 23 (page 126), lists the components of these media, but it is not evident from this why the differences obtained should have arisen. TSA was consistently shown to be effective in recovering survivors, especially in the later stages of an experiment. Moss and Speck (207) showed that a proportion of the surviving population of E. coli that had been stored at -20°C for up to 28 days, was unable to grow on minimal agar medium, but could develop on TSA. Trypticase was found to be the component responsible for the recovery of injured cells and they were able to identify five peptides that were mainly responsible for this activity. They suggested that the peptide could stimulate cell repair and growth during the lag phase, thereby, preventing death; trypticase could play a similar role here.

It was not considered important to use the defined media + agar as plating media for recovery of surviving organisms, because it is unnecessary for these to be of identical nutrient status.

An interesting change in colonial morphology was observed with C. albicans survivors that grew on MEA (Table 24d, page 134). This change in colony shape was observed after about 3 hours contact with thiomersal and subsequently occurred on all surviving colonies. As it was not found with colonies grown from organisms in buffer only, it must have arisen as a result of damage done by thiomersal to C. albicans. It is not clear, however, why it only occurred on this media type.

These experiments were carried out at 25°C and with an initial challenge size of 10^6 cfu ml^{-1}. This inoculum size has been criticised

on occasions, for being too high a challenge dose, unrealistic of 'in-use' situations. The results obtained with a single concentration of thiomersal (0.008% w/v) and different initial inoculum sizes are presented in Figures 57-61 (pages 175-179). It was found that whilst, the highest inoculum level tested (10^7 cfu ml^{-1}), survived the longest, at levels of 10^6 and below, there were only small differences between the $t_{0.1}$ values obtained (Table 32, page 180) with Staph. aureus, Ps. aeruginosa and E. coli. With the lowest inoculum level used, namely, 10^3 cfu ml^{-1}, surviving organisms were still detected at 64 hours for Staph. aureus, 5 hours for Ps. aeruginosa, 6 hours for E. coli, and 2 hours for C. albicans and B. subtilis. It can, therefore, be seen that even with lower challenge levels 'killing' is not achieved within 6 hours for Staph. aureus and E. coli so that with multidose preparations, the potential risk of infection from solutions contaminated with these organisms would be high.

The influence of temperature on the antimicrobial activity of thiomersal is presented in Figures 47-51 (pages 160-164). It is evident that for all organisms, a reduction in temperature is accompanied by a decrease in activity of thiomersal, apart from with E. coli, where at 15°C, death occurred faster than at 20°C (Figure 49, page 162). E. coli are known to be sensitive to cold shock (208), and it may be that this has contributed to the increased kill observed. The reduction in antimicrobial activity was most evident with Staph. aureus, where at 15°C, it was so reduced that at 10 days, only about a 10% drop in viable count was obtained. Table 30 (page 166) lists the calculated Q_{10} values and it can be seen that these varied with the test organism as has been suggested (166). An average value of about 2 was obtained which is in keeping with the Q_{10} value of 2 quoted for many chemical

and biological reactions (196).

The experiments were carried out with cells in late stationary phase, where active growth is known to have ceased. The cells are probably degrading their endogenous reserves of protein and RNA to derive the energy for maintenance of minimum activity, or living off the exudates from cells that have died. The prolific enzyme activity seen in exponentially-growing cultures will, therefore, have ceased, but the 10°C drop in temperature, for example from 25°C to 15°C, will have resulted in a halving of the rates of reaction of any enzyme systems operating. The net effect of such a temperature drop whilst lowering the chances of a thiomersal molecule 'hitting' a bacterial cell, would also result in its having a much reduced effect on any enzyme system in operation when it penetrated, because these would also all be functioning at reduced levels. A drop in temperature would also have a stabilising effect on thiomersal so that less degradation would occur.

The results again highlight the ineffectiveness of thiomersal as an antimicrobial agent because at about room temperature, in temperate climates i.e. 20°C, a 0.008% w/v solution did not succeed in reducing the viable count of the test organisms by three log cycles within 6 hours (Table 29, page 165). The antimicrobial activity of solutions preserved with even lower concentrations of thiomersal, for example contact lens solutions, would be even further reduced and the use of this preservative by itself, in such products, must be questioned.

It seemed interesting to determine the antimicrobial effectiveness of thiomersal at different stages of the cell cycle, to try to relate

this to anticipated differences in enzyme activity and cell wall thickness. Cultures were , therefore, harvested in early and late exponential phase and used in challenge experiments. The results of these experiments are presented in figures 52-56 (pages 168-172). As anticipated, cells from exponential phase cultures were found to be more sensitive to thiomersal than were those in stationary phase. In all cases, a pronounced reduction in the shoulder period of the curves was apparent in cells from 'young' cultures. With Ps. aeruginosa and E. coli, a very reduced shoulder and no shoulder respectively, were evident when both early exponential and late exponential/early stationary cells were used (Figures 53 & 54, pages 169 & 170). This suggests that whatever is responsible for the shoulder obtained with late stationary phase cells, is formed between early and late stationary phase. One of the possible reasons for this is that cell walls are reported to be thinner in exponential phase cells than in stationary phase cells (148). The rate of growth could also be of some significance. In general, when bacteria grow faster, they are larger and contain a high amount of RNA (209). The average number of nuclear bodies per bacterium has been shown to be greater at faster growth rates for a reason that is somehow related to the process of cell division. Bacteria growing slowly have a higher content (per unit mass) of cell membrane and cell wall than fast-growing bacteria, so that at fast growth rates, synthesis of these cell envelopes could be limiting. The cells could then adjust to the relatively smaller amount of envelope material by an increase in volume and hence a decrease in the ratio of surface to volume. At a sub-cellular level, the cell mass per nucleus varies mainly because of variations in ribosome content (209), and this has been shown to vary with the growth rate, from a very low value in slow growing cultures to one third or more of the dry weight of fast-growing organisms. The

number of ribosomes has also been shown to be directly proportional to the rate of growth (209), which in balanced growth, also means that the number of ribosomes is directly proportional to the rate of protein synthesis. In exponentially growing cultures, therefore, enzyme activity is at a maximum. These reactions may be thought of as occurring in three classes:-

1) Class I reactions - these are degradative reactions which break down glucose to aliphatic carbon compounds; the net process results in energy,
2) Class II reactions - these are biosynthetic reactions which use the carbon skeletons and energy supplied from class I reaction to form the small molecules which are the basic components of the macromolecules,
3) Class III reactions - these are biosynthetic reactions which convert the basic small molecules into macromolecules; when sufficient numbers of these have been synthesised, the cell divides.

Thiomersal thus has an increased effect, because not only is it easier for it to penetrate the thinner cell walls, but once it has done so, it can affect sulphydryl-containing enzymes functioning at a number of possible stages and so stop cell division, ultimately causing death.

Another factor that may contribute to the increased sensitivity of exponential phase cells is the reduced amount of lipid present in the cell walls of such cells (149, 150). Thiomersal is poorly lipid-soluble so that this may have some significance here. Lipid enhancement has been shown to markedly increase the resistance of Gram-positive bacteria to certain penicillins (210). Gram-positive bacteria normally contain

very little or no lipid, however, so that the great resistance of Staph. aureus to thiomersal must be due to some other factor(s).

The experiments discussed above were carried out using solutions of thiomersal prepared in isotonic Sörensen's phosphate buffer at pH 7.0. It was decided to investigate the influence of the composition of the test solution on the antimicrobial activity of thiomersal, so distilled water was considered as an alternative. This was boiled to drive off dissolved carbon dioxide before being sterilised for use, as the pH of laboratory distilled water was found to vary between pH 4.5-5.7; such a range of pH would have complicated the interpretation of results. After boiling and cooling, the pH of the distilled water was found to be about pH 6.5, which was considered acceptable. It was decided to test the activity of thiomersal prepared in the appropriate chemically-defined media as the organisms would suffer a minimum of stress and should even be able to grow slowly in the absence of thiomersal, at 25°C. Thiomersal would thus be exerting an effect on cells that had a supply of nutrients and the experimental conditions in which to grow, and it was considered that this might yield some interesting data.

The results of these experiments are presented in Figures 31-35 (pages 138-142), and it can be seen that the ability of cells to survive exposure to thiomersal is strongly influenced by the composition of the solution in which it is formulated. Table 26 (page 144) depicts the $t_{0.1}$ values obtained and it is apparent that once again, these values are well outside six hours for Staph. aureus, being about 24h, 67h and 39h for distilled water, phosphate buffer and growth medium, respectively. Formulating thiomersal in distilled water, therefore, resulted in a considerable reduction in the $t_{0.1}$ value obtained for

this organism. Allwood and Russell (211) showed that when aqueous suspensions of Staph. aureus were stored at different temperatures up to 55°C, there was always a decrease in optical density readings made at 500nm over the six hour period they tested. This decrease in optical density was shown to be at least partially related to the increase in the amount of 260nm-absorbing material leaked from the cells, and it was also related to changes in the refractive index of the cells. These findings may have contributed to the increased sensitivity of Staph. aureus obtained when thiomersal was prepared in distilled water. Staph. aureus was found to survive extremely well in distilled water over the duration of the experiment, as indeed did Ps. aeruginosa, E. coli and C. albicans; only with B. subtilis was a drop of about three log cycles obtained at 48h (Figure 36a, page 143). These results contrast with the findings of others (59, 160-163), where distilled water was shown to be an unfavourable diluent. In all these cited cases, however, the test organisms had come from extremely rich nutrient backgrounds, so that it would appear that growing an organism in a chemically-defined medium increases its ability to survive in unfavourable environments, such as distilled water. The sensitivity of B. subtilis to distilled water and other buffer formulations, may be related to the fact that this organism is a spore-former and there would be little need for its vegetative form to be able to survive unfavourable environments.

With Ps. aeruginosa (Table 26, page 144), t_{10} values of about 7h, 4h and 6h, were obtained for distilled water, phosphate buffer and growth medium, respectively. Extended shoulders were evident in the curves obtained for thiomersal in distilled water and growth medium against this organism (Figure 32, page 139), and only very slow kills followed.

With E. coli, t_{10} values of about 4h, 4h and 6h, were obtained when thiomersal was prepared in distilled water, phosphate buffer and growth medium, respectively. The cell membranes of Ps. aeruginosa and E. coli have been demonstrated to have different permeability barriers (213), and this may account for the difference in sensitivity of these two organisms, seen when thiomersal was prepared in distilled water.

The results obtained for C. albicans were quite unusual. When thiomersal was prepared in distilled water, it had no appreciable effect on this organism, despite a 32-hour contact time; when prepared in the growth medium, however, a $t_{0.1}$ value of about 5h was recorded, compared with a value of about 8h when it was prepared in phosphate buffer. It might appear then, that thiomersal was especially effective against dividing yeast cells, because studies on the survival of all test organisms in growth media over the course of the challenge experiments revealed that growth did indeed occur in the absence of thiomersal (Figure 36b, page 143). It had been anticipated that an increased activity would be found in solutions of thiomersal formulated in the growth media, because the cells would have the potential to divide. The fact that this was not found in all cases emphasises the complexity of the reactions involved before death can be induced by this compound.

One factor which might have had some influence in these experiments was the difference in tonicity of the three solutions. The effect of tonicity can be studied separately, and this was done as shown in Figures 37-41 (pages 146-150). These show that there was only a marginal effect on the activity of thiomersal against Ps. aeruginosa and B. subtilis. Both hypotonic and hypertonic solutions containing thiomersal had a very similar effect on Staph. aureus, and $t_{0.1}$ values of about 50h were

obtained compared with that of about 67h, when thiomersal was prepared in isotonic solution (Table 27, page 151). With E. coli, however, an isotonic solution was found to be the most effective, followed by a hypertonic and then a hypotonic solution; here, $t_{0.1}$ values of about 6h, 8h and 20h, respectively, were obtained. Against C. albicans, hypertonic and isotonic solutions had very similar effects, with $t_{0.1}$ values of about 6 and 7 hours respectively, whereas a hypotonic solution had a $t_{0.1}$ value of 36 hours. The slow kill obtained in the latter formulation does parallel the even slower kill obtained when thiomersal was prepared in distilled water and used against this organism. Thus, tonicity appears to have a major effect on the activity of thiomersal against this yeast. The effect is not a sensitivity to sodium chloride, as is known to occur with certain micro-organisms (213), because the yeast survived well in hypertonic buffer over the duration of the experiment. B. subtilis was, however, demonstrated to be quite salt-sensitive. In hypotonic buffer, this organism only dropped about one log cycle in viable count by day 14, whereas in hypertonic buffer, the same drop occurred after about 6 hours (Figure 41, page 150).

The variability of the effects of tonicity serves to reinforce the complexity of the antimicrobial activity of thiomersal, and it is unclear how the observed differences in rates of kill can be accounted for purely in terms of differences in tonicity. Chloride ions are known to increase the breakdown of thiomersal (personal communication from Dr. T. Jones of the Wellcome Foundation, 1980, and Dr. S. Eriksen of Allergan, 1981), so that these could exert an influence here.

Eye-drop formulations vary markedly in their pH and so the influence of this on the antimicrobial activity of thiomersal is important in

practice. This was studied over the pH range thought to be of practical significance, namely, 4.5 to 8.0. It was found that this range could be achieved with a single buffer system, Sörensen's phosphate buffer, by omitting the di-sodium hydrogen orthophosphate dihydrate, for the lowest pH of 4.5. The results of these experiments are presented in Figures 42-46 (pages 153-157). From these, it can be seen that the effect of pH is rather complex. At first sight, it would appear that when prepared at pH 4.5, all solutions showed an enhanced activity. This was especially noticeable with Staph. aureus where a $t_{0.1}$ value of about 8h was recorded compared with that of about 67h at pH 7.0 (Table 28, page 158). The survivor curve for pH 4.5 in figure 42 (page 153), was obtained by plotting the surviving organisms as a percentage of those found at time '0' in the challenge experiment. Staphylococci are sensitive to this low pH as, in the absence of thiomersal, a drop of over one log cycle occurred in 10 hours. To allow for this, the data were plotted again, as a percentage of the viability found at each sampling time in the pH 4.5 buffer without thiomersal (Figure 89, page 244). In this new curve, represented in Figure 89 by closed triangles, an extended shoulder period lasting about 8 hours can be seen, during which, a drop in viable count of about one and a half log cycles is obtained; a rapid drop in numbers is not seen until after 8 hours though and no surviving organisms were detected in the test solution at 12 hours. The contribution made by pH alone (closed circles), to the observed kill can now be appreciated, so that it is not, as first appeared, entirely due to thiomersal being more effective at acid pH. Thiomersal has a pk_a of 3.04 (101), so that at a pH of 4.5, some thiomersal will occur in the highly lipid-soluble acid form. An increase in pH generally resulted in a decrease in anti-microbial activity which is in agreement with the findings of other

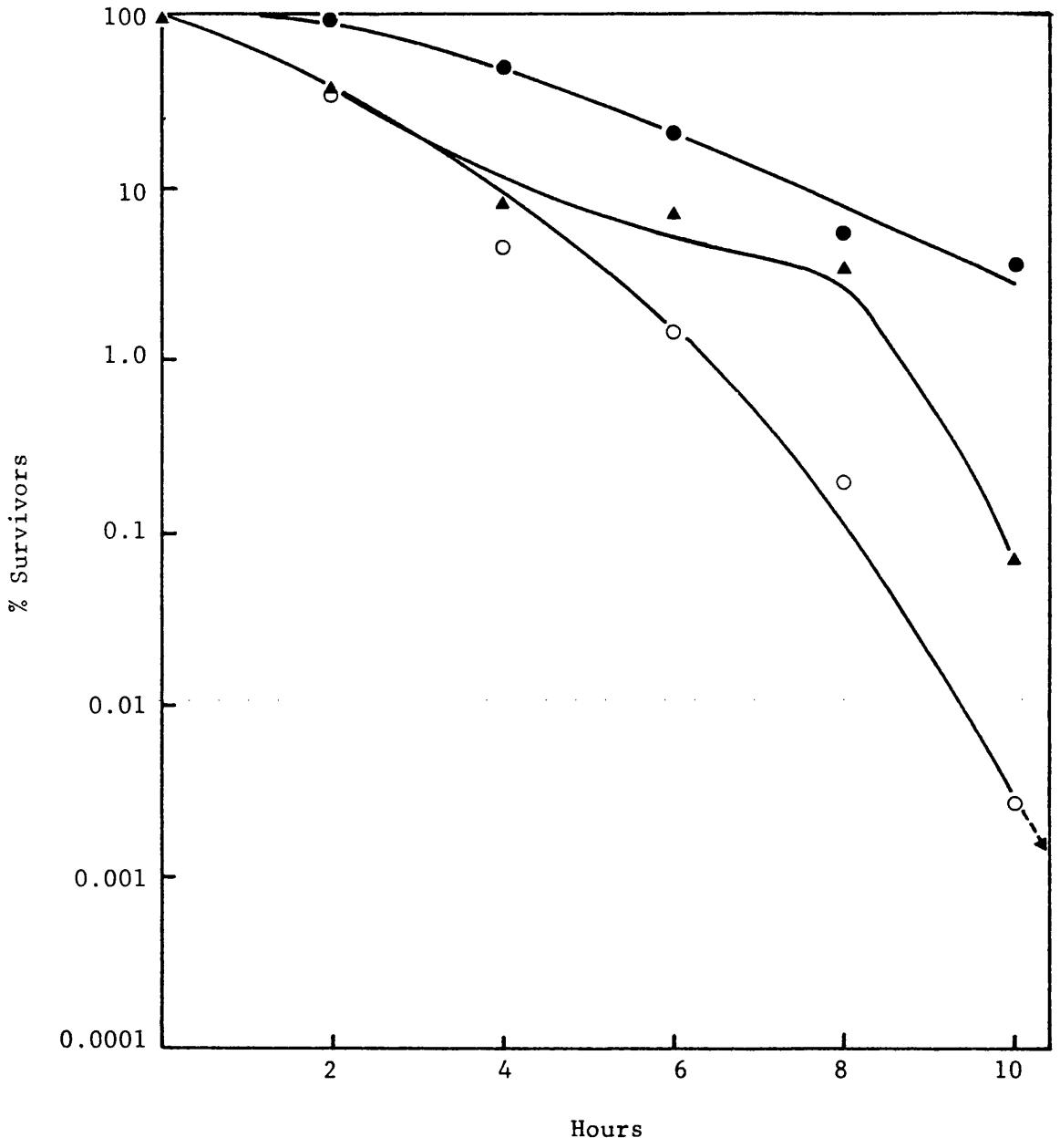


Fig.89 Effect of 0.008% w/v thiomersal in isotonic Sörensen's phosphate buffer, pH 4.5, at 25°C, on Staph. aureus (○).

(●) Survival in pH 4.5 buffer

(▲) Survival of Staph. aureus in thiomersal, plotted as % of survival in buffer at each sampling time

workers (171). The exceptions to this were with Staph. aureus and Ps. aeruginosa, where solutions at pH 8.0 were more effective than at pH 7.0. A reason for the enhanced activity obtained here could arise from the proposal made by Davisson et al (74), that the $C_2H_5Hg^+$ ion is responsible for the antimicrobial activity of thiomersal; at alkaline pHs, any $C_2H_5Hg^+$ would be bound to a greater extent by the negatively charged bacterial cells and would consequently have an enhanced effect. If this were the only reason, however, it is strange that an increase in antimicrobial activity was not found when test solutions at pH 8.0 were used against the other test organisms. Polyvalent phosphates are chelating agents, and an increase in pH will increase the amount of the di-sodium hydrogen orthophosphate dihydrate present. By chelating with any trace metals present possibly as contaminants from the buffer salts, this could stabilise the thiomersal solutions and so inhibit degradation. At the same time, the chelating influence could also have an effect on the structural integrity of the test organisms. The possibility that different active moieties may be responsible for the observed differences in kill with the various test organisms should also be considered.

The final section of this work deals with the effect of photodegradation on the antimicrobial activity of thiomersal. The best evidence that some changes might have to occur to thiomersal to make it biologically active is in fact provided by these experiments. Figures 62-67 (pages 182-187), show that degraded solutions are always more effective than undegraded. With all the test organisms, a reduction in the shoulder period of the survivor curves was obtained when degraded solutions were used. This was especially noticeable with Ps. aeruginosa, E. coli and C. albicans (Figures 63, 65 and 66, pages 183, 185 and 186).

With Staph. aureus, E. coli and B. subtilis, the close proximity of these curves suggests that the degradation product(s) responsible for the increased kill, has/have been formed within two days. An increased holding time in the light box, therefore, did not result in an increased kill. With Ps. aeruginosa, however, activity was found to increase with a corresponding increase in photodegradation time, with a maximum being reached at about Day 8. This might suggest that a product or products formed during this period has/have a marked antimicrobial activity against this organism. The effect was obviously not characteristic of Gram-negative organisms, as it had not been found with E. coli. A second strain, the disinfectant testing strain, Ps. aeruginosa var. erythrogenes, NCTC 6749, was, therefore, tested with degraded and undegraded thiomersal. Figure 64 (page 184) shows that this strain has a similar sensitivity pattern to that of Ps. aeruginosa, NCTC 6750, namely, that with an increase in photodegradation, there is a corresponding increase in kill and decrease in shoulder of the curves obtained.

When reference is made to the $t_{0.1}$ values recorded in Table 33 (page 188), the vastly superior antimicrobial activity of degraded solutions is apparent. This is especially noticeable with Ps. aeruginosa, NCTC 6750, where a ten-fold reduction in the $t_{1.0}$ value is seen with 8 and 10 day-degraded solutions, when compared with that of an undegraded solution. With Ps. aeruginosa var. erythrogenes, this ten-fold reduction in the $t_{1.0}$ value is seen by Day 6 and a forty-fold reduction is evident by Day 10. The uniqueness of this effect for strains of Ps. aeruginosa, as opposed to Gram-negative bacteria represented by E. coli, must be a reflection of the different permeability characterisitcs of the membranes of these two organisms.

With C. albicans (Figure 66, page 186), the picture is slightly more complex. Here, the effective antimicrobial compound has again been formed by Day 2, so that the kill obtained with a Day 4 solution is similar but slightly reduced; $t_{0.1}$ values of about 1.5h were obtained for these curves (Table 33, page 188). With Day 6 and Day 8 solutions, however, a drop in antimicrobial activity was apparent; here, $t_{0.1}$ values of between 2.5-3.0h were recorded. The Day 10 solution was found to have an activity between these two sets of data, and a $t_{0.1}$ value of about 2h was obtained. These results appear to suggest that that at Days 6 and 8, the active moiety formed in the early stages of photodegradation, has itself degraded to give a product with reduced activity, or that, in degrading, it is present at a lower concentration and, therefore, has a reduced effect; at Day 10, degradation of the active moiety may have resulted in a the formation of a further product with an increased activity as compared with Days 6 and 8, hence the increased kill observed.

It is known that the photochemical degradation of drugs is complex, because of the numerous secondary reactions that can follow light absorption by drugs; photochemical products of a reaction may themselves undergo a further series of chemical reactions, sometimes with the parent molecule. HPLC analyses of these solutions (Figures 79a-83a, pages 208-212), show the variety of degradation products that have been formed. The numbers of degradation products detected are far more than have previously been found in other assay systems and may be partially accounted for by the report that chloride ions may promote the degradation of thiomersal (page 242).

Since it has been demonstrated that degraded solutions are markedly

more active against the test organisms, the chelating agent, EDTA, was included in test formulation in an attempt to determine if chelateable ions are involved in the enhanced activity observed experimentally. EDTA is frequently included in ophthalmic preparations containing thiomersal at 0.01% and 0.1% w/v, apparently to improve shelf life (Dr. S. Eriksen, personal communication, 1981). It was, therefore, decided to use these concentrations in the test formulations. The effect of EDTA on undegraded thiomersal solutions is shown in Figures 90-94 (pages 249-253). These show that the effect varied with the test organism. With Staph. aureus, EDTA apparently decreased the antimicrobial activity of thiomersal so that increased t_{10} values were obtained (Table 40, page 254). The effect appeared to be concentration dependant. This result confirmed the findings of Richards and McBride (46), that EDTA-antibacterial combinations were not always more effective. With Ps. aeruginosa, however, the incorporation of EDTA in thiomersal solutions resulted in an enhancement of activity (Figure 91, page 250), and $t_{0.1}$ values were halved, regardless of the EDTA concentration. EDTA was found to antagonise the effect of thiomersal, when used against E. coli (Figure 92, page 251), so that once again, the effect seen with Ps. aeruginosa was not characteristic of Gram-negative organisms.

The effect of EDTA on the cell wall of Gram-negative organisms has been the subject of several detailed studies (214-222). E. coli treated with EDTA was shown, under certain conditions, to become permeable to several unrelated molecules, to which it was normally impermeable (218). It is accepted that EDTA can cause extensive damage to the cell wall of Ps. aeruginosa (215, 216), the release of intracellular solutes (212, 217), and death of this organism (216, 217). Sensitivity to EDTA has

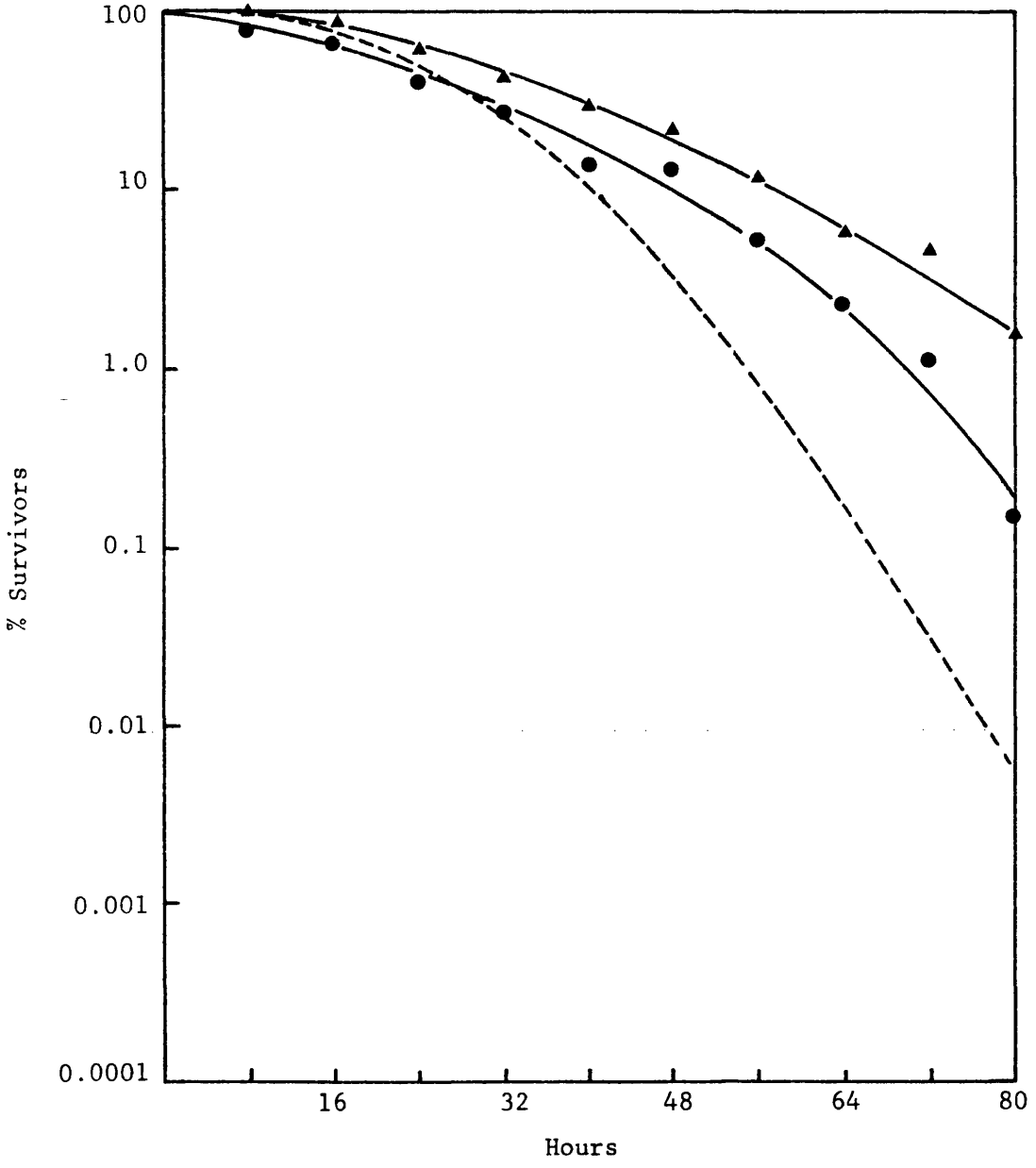


Fig.90 Effect of 0.008% w/v thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0, without EDTA (---), with 0.01% w/v EDTA (●) and with 0.1% w/v EDTA (▲), at 25°C on Staph. aureus.

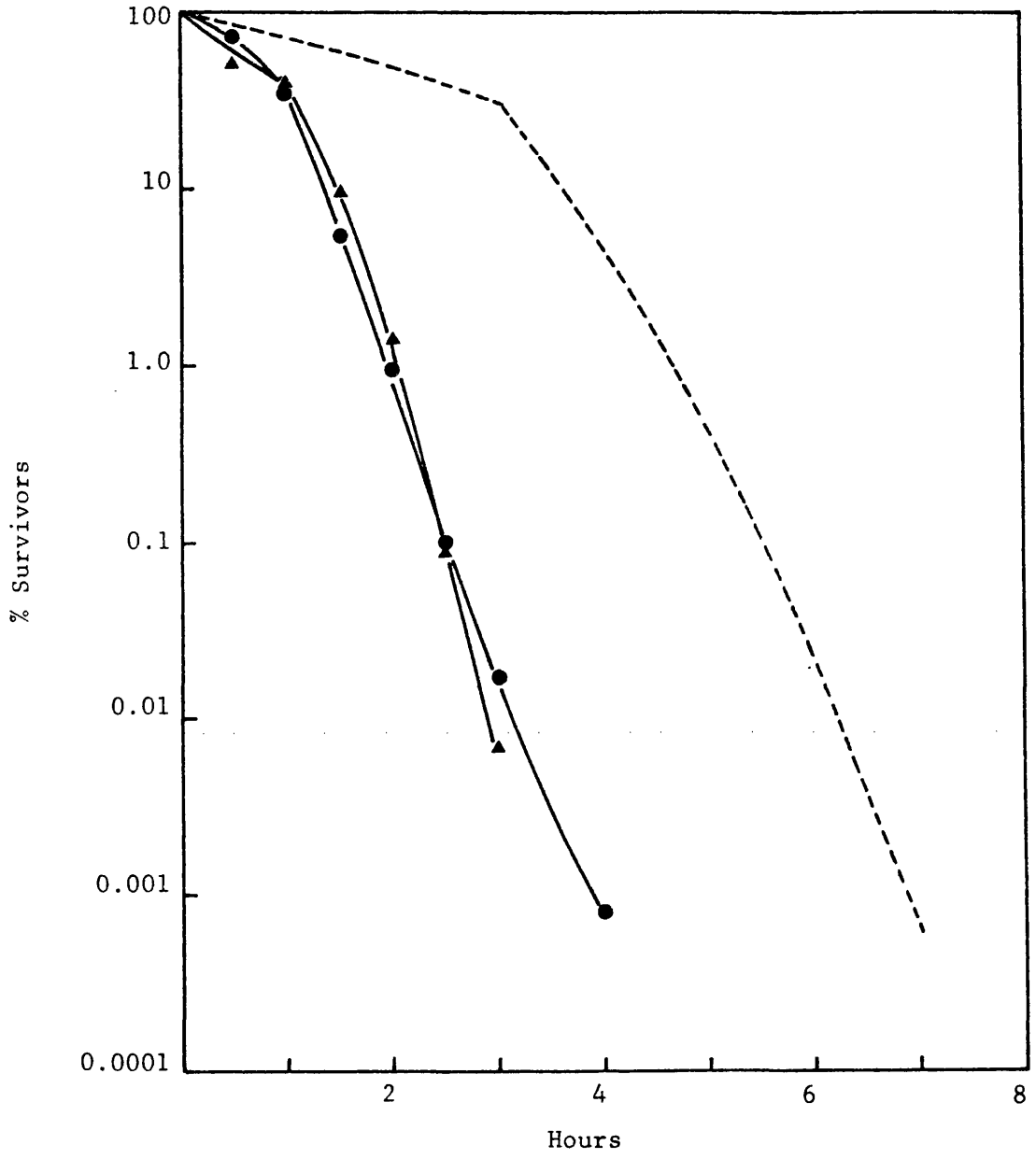


Fig.91 Effect of 0.008% w/v thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0, without EDTA (---), with 0.01% w/v EDTA (●) and with 0.1% w/v EDTA (▲), at 25°C on Ps. aeruginosa.

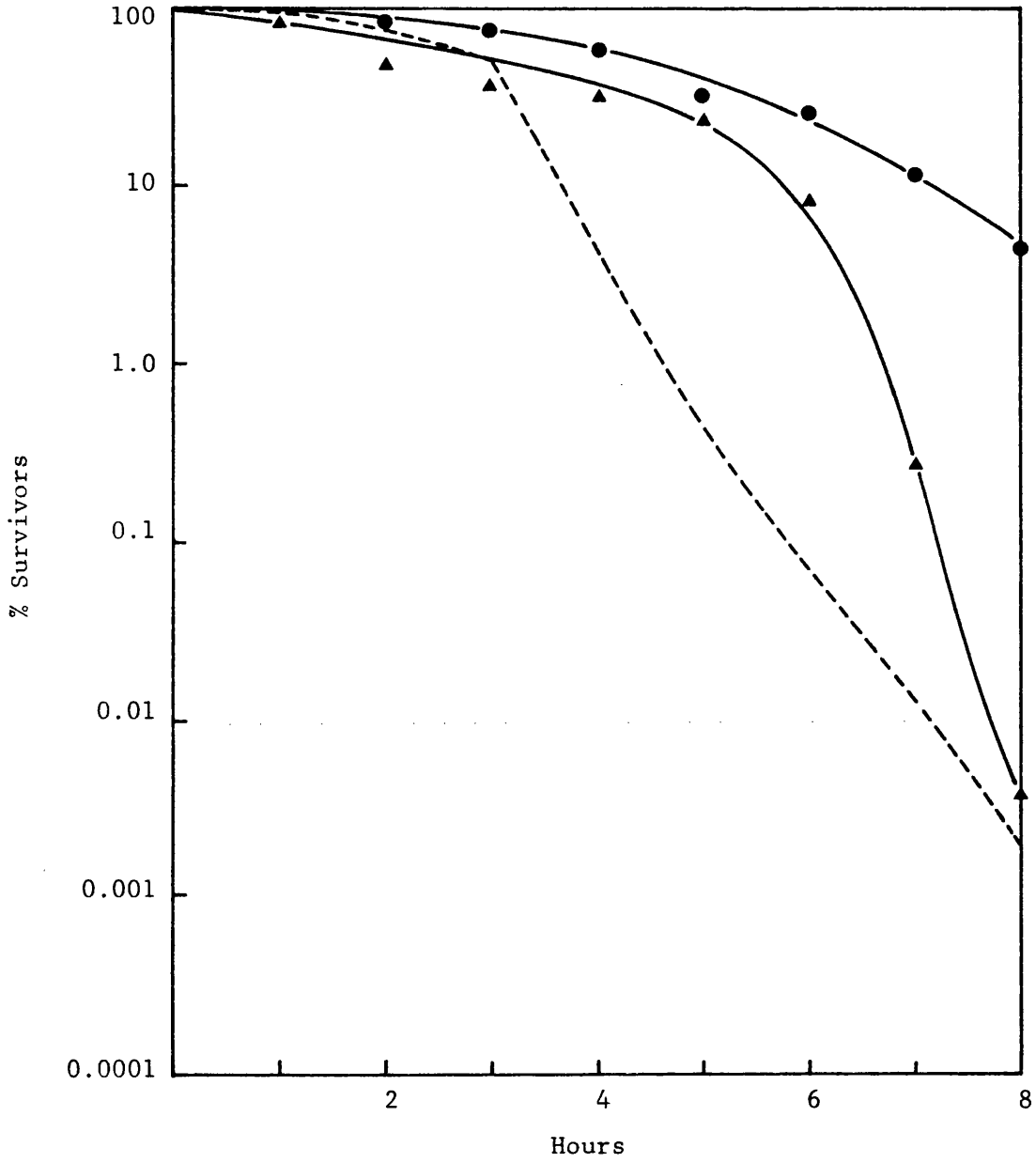


Fig.92 Effect of 0.008% w/v thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0, without EDTA (---), with 0.01% w/v EDTA (●) and with 0.1% w/v EDTA (▲), at 25°C on E. coli.

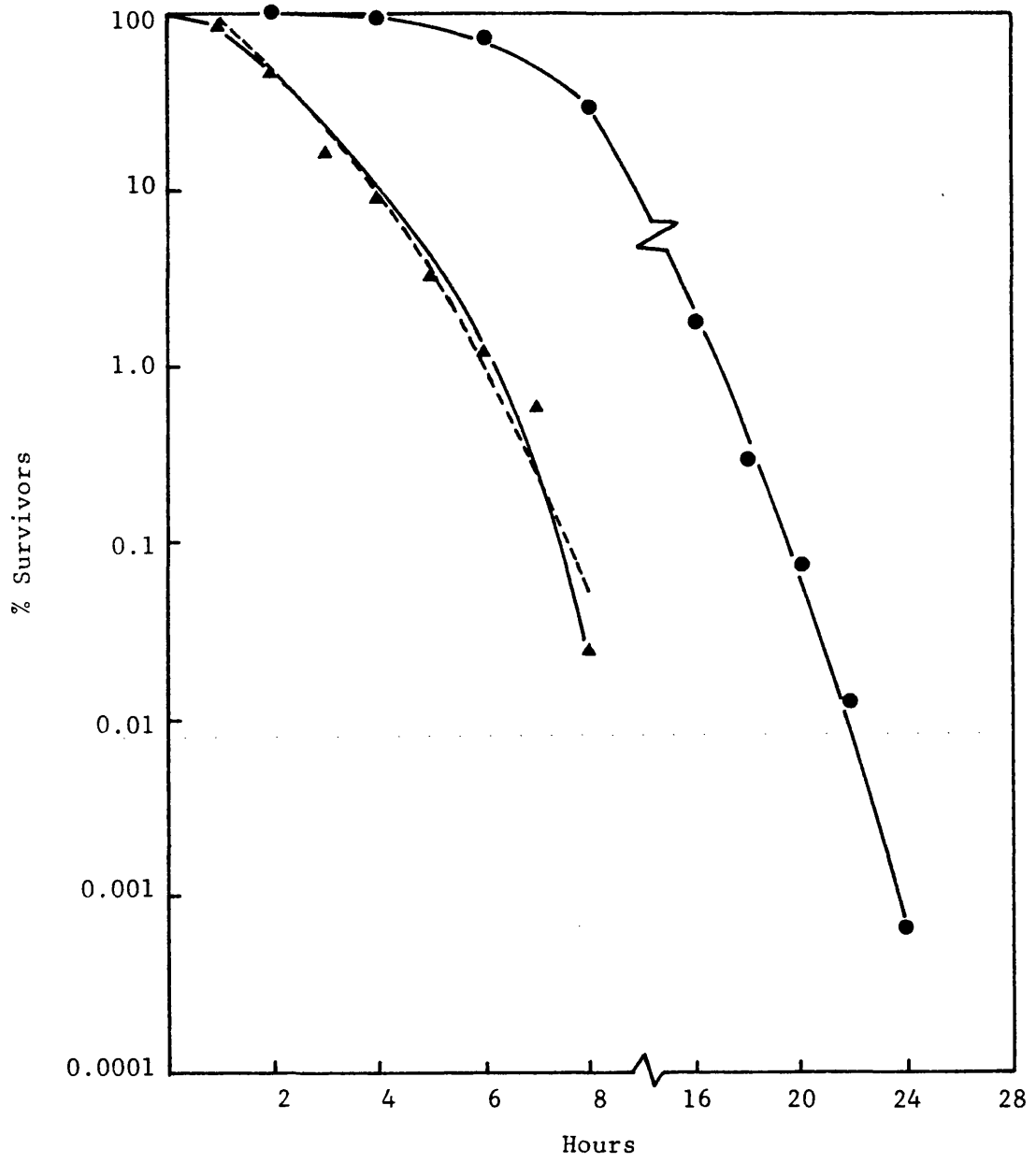


Fig.93 Effect of 0.008% w/v thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0, without EDTA (---), with 0.01% w/v EDTA (●) and with 0.1% w/v EDTA (▲), at 25°C on C. albicans.

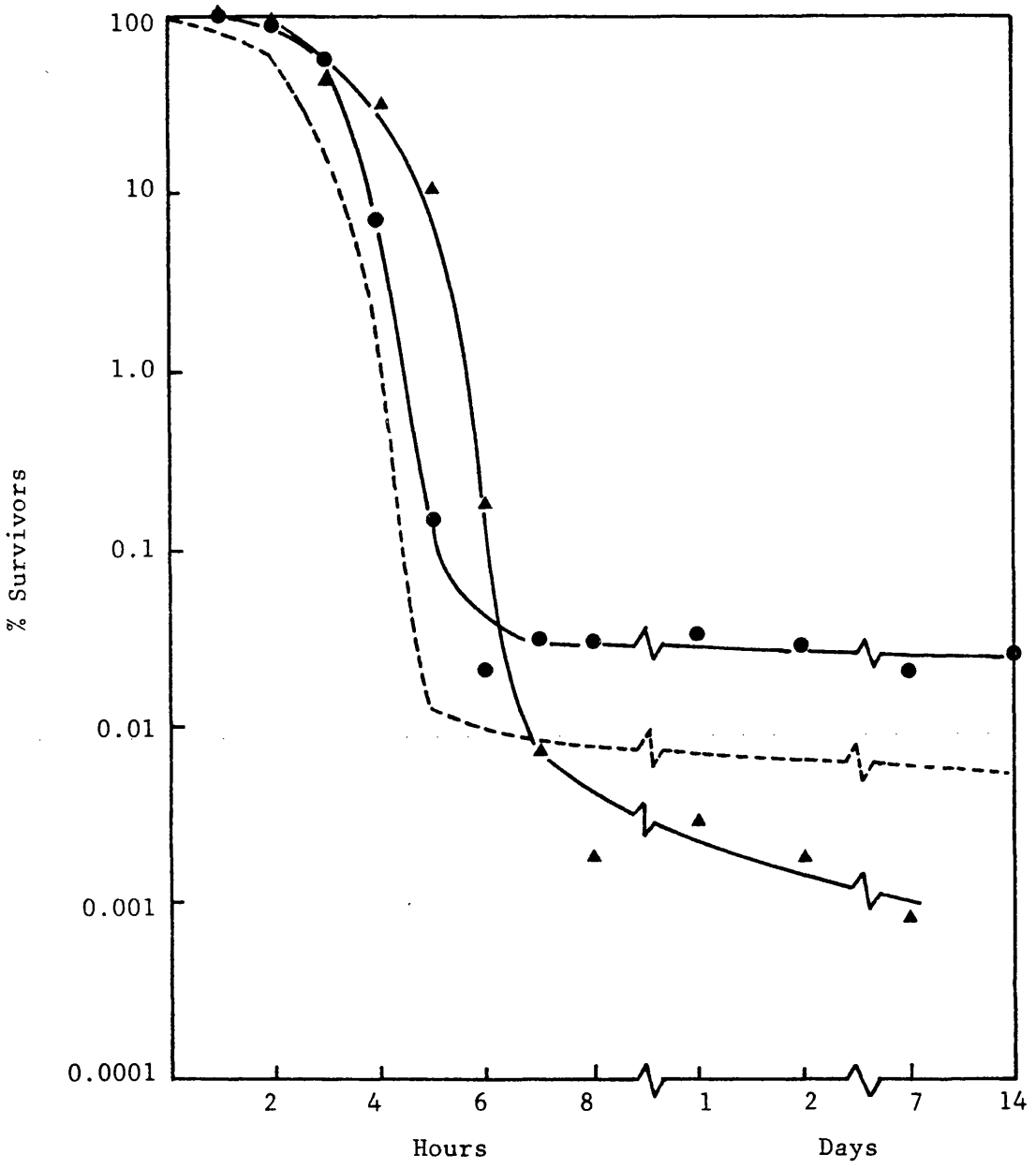


Fig.94 Effect of 0.008% w/v thiomersal in isotonic Sörensen's phosphate buffer, pH 7.0, without EDTA (---), with 0.01% w/v EDTA (●) and with 0.1% w/v EDTA (▲), at 25°C on B. subtilis.

TABLE 40

$\overset{+}{+}t_{0.1}$ values recorded from the effect of EDTA at 0.01% w/v and 0.1% w/v on the antimicrobial activity of undegraded thiomersal.

EDTA (% w/v)	$t_{0.1}$ values (hours) $\overset{+}{+}$Staph. aureus	Ps. aeruginosa	E. coli	C. albicans	B. subtilis
0.01	48.0	2.50	8-16	19.5	5.1
0.1	58.0	2.48	7.25	7.6	6.2

$\overset{+}{+}t_{10}$ values read for Staph. aureus.

even been suggested as a possible characteristic in the taxonomy of pseudomonads (214, 220). A difference in sensitivity to EDTA between E. coli and Ps. aeruginosa was not unexpected, because of known differences in membrane permeability. Matsushita et al (222) attempted to isolate and characterise the outer and inner membrane of Ps. aeruginosa and looked at the effect of EDTA on the cell envelope. They confirmed that a number of proteins and a protein-lipopolysaccharide complex were released from the cell envelope of Ps. aeruginosa, after treatment with EDTA (5mM and 10mM). This was also found to occur with E. coli, but unlike Ps. aeruginosa, E. coli did not lyse after treatment with EDTA. Apparently, the total disintegration of the outer membrane caused by the release of specific components is not as drastic in E. coli as in Ps. aeruginosa. Thus, divalent metals appear to play a more important role in the structural intergrity of the outer membrane of Ps. aeruginosa than in E. coli.

If the results obtained are reviewed in the light of these findings, then the enhanced activity of thiomersal solutions incorporating EDTA against Ps. aeruginosa, is understandable. This effect obviously outweighs any chelating effect exerted by EDTA on thiomersal, and an increased kill is obtained. With E. coli and indeed with the Gram-positive bacteria, EDTA-thiomersal combinations are less effective, presumably because less chelateable degradation products of thiomersal are available for antimicrobial activity. With C. albicans, a rather strange result was obtained (Figure 93, page 252). The solution with 0.1% w/v EDTA and that without EDTA, had rather similar activities, and were about twice as effective as the solution with 0.01% w/v EDTA. Why this should have happened is unclear; a possible explanation is that the lower concentration removes chelateable material, causing a

decrease in sensitivity, as would be expected. The higher concentration of EDTA, as well as chelating , may somehow affect the yeast by possibly damaging the cell wall. The two opposing effects could then cancel each other out.

The effect of EDTA on the photodegradation of thiomersal is rather complex. In Table 41, page 257, the t_{10} values obtained from Figures 62, 63 and 65-77 (pages 182, 183, 190-194 and 197-201), have been recorded so that a direct comparison can be made between all the test organisms. The relevant data from these Figures has also been represented diagrammatically in Figures 95-99 (pages 258 to 262), to highlight the salient features of the original figures. These results are now discussed for each individual organism.

Staph. aureus

In Figure 95A, it can be seen that degraded solutions with EDTA at 0.01% w/v (D8b and D10b), are more effective than their undegraded counterparts, shown here as a dash-dot line. As the curves for Days 2-8 were very close together (Figure 68, page 190), only the data for Day 8 were included in Figure 95A. The close proximity of these curves suggests that, once again, the active degradation product(s) has/have been formed by Day 2; a slight increase in activity with the Day 10 solution was observed. It can also be seen in Figure 95A that Days 2-8 degraded solutions with 0.01% w/v EDTA (represented by the D8b line), are only marginally more effective than an undegraded solution of thiomersal in buffer only (represented by a dashed line), especially towards the later stages of the survivor curves. An attempt was made to relate these findings with the HPLC chromatograms of degraded solutions with 0.01% w/v EDTA (Figures 79b-83b, pages 208-212). As no

TABLE 41

t_{10} values obtained from the antimicrobial activity of photochemically degraded solutions of thiomersal in buffer without EDTA, with EDTA at 0.01% w/v and with EDTA at 0.1% w/v.

	Days in Light Box																	
	0			2			4			6			8			10		
		EDTA			EDTA			EDTA			EDTA			EDTA			EDTA	
Organism	0	0.01	0.1	0	0.01	0.1	0	0.01	0.1	0	0.01	0.1	0	0.01	0.1	0	0.01	0.1
Staph. aureus	**40**	48	58	**28**	32	28	**28**	32	29	**28**	30	46	**32**	30	53	**32**	28	33
Ps. aeruginosa	**4**	1.5	1.5	**1**	0.5	0.5	**0.75**	0.5	0.5	**0.5**	0.5	0.75	**0.25**	0.5	0.75	**0.25**	0.5	1
E. coli	**3.5**	7	6	**2**	3.5	3.5	**2**	3.5	3.5	**2**	3.5	3.5	**2**	3.5	3.5	**2**	3.5	3.5
C. albicans	**4**	8-16	4	**1**	3	2	**1**	3	2	**1**	3	2	**1**	2.5	2.5	**1**	2	2.5
B. subtilis	**3**	5	4	**2**	4	3	**2**	4	3	**2**	3.5	3	**2**	3.5	2	**3**	3.5	3

All the above t_{10} values are approximate data, read in hours

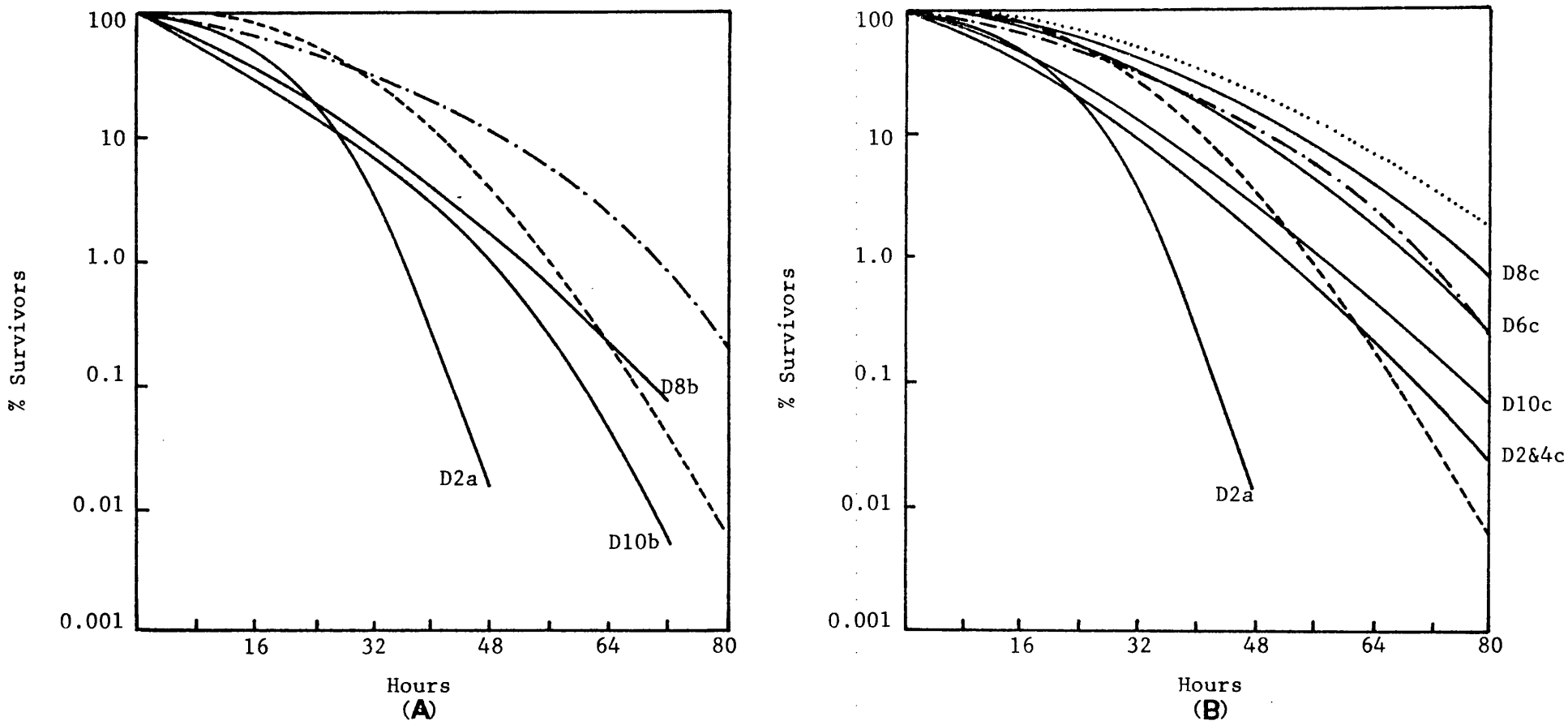


Fig.95(A & B) Effect of photodegradation on the antimicrobial activity of thiomersal against Staph.aureus.

(---) Undegraded solution in buffer only

(-·-) Undegraded solution in buffer with 0.01% w/v EDTA

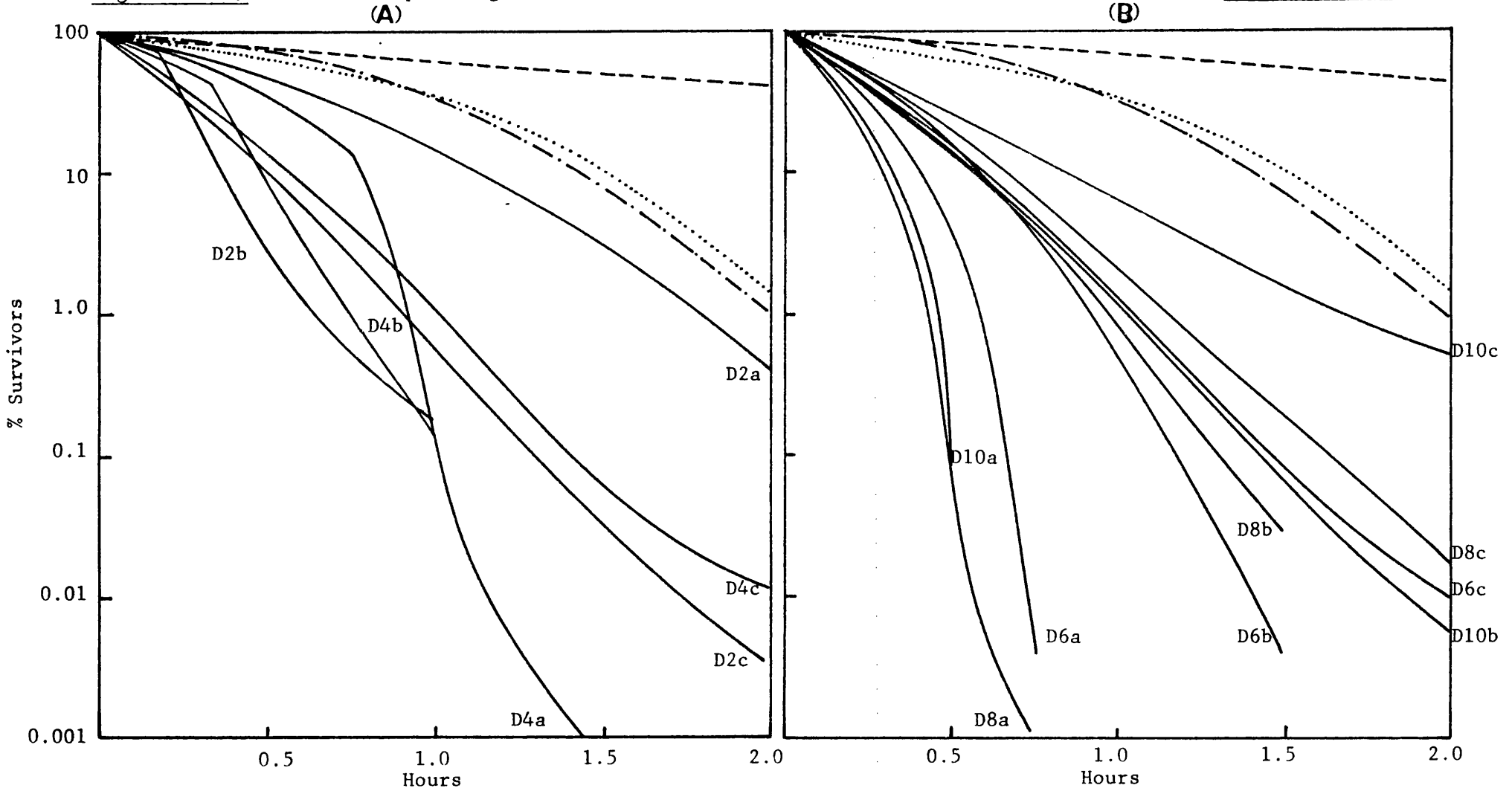
(····) Undegraded solution in buffer with 0.1% w/v EDTA

D2a Day-2 degraded solution in buffer only

D8b,10b Days 8 and 10 degraded solutions in buffer with 0.01% w/v EDTA

D2c-D10c Days 2-10 degraded solution in buffer with 0.1% w/v EDTA

Fig.96 (A & B) Effect of photodegradation on the antimicrobial activity of thiomersal against Ps. aeruginosa.



(---) Undegraded solution in buffer only

(·—·) Undegraded solution in buffer with 0.01% w/v EDTA

(···) Undegraded solution in buffer with 0.1% w/v EDTA

D2a-D10a Days 2, 4, 6, 8 and 10 degraded solutions in buffer

D2b-D10b Days 2, 4, 6, 8 and 10 degraded solutions in buffer with 0.01% w/v EDTA

D2c-D10c Days 2, 4, 6, 8 and 10 degraded solutions in buffer with 0.1% w/v EDTA

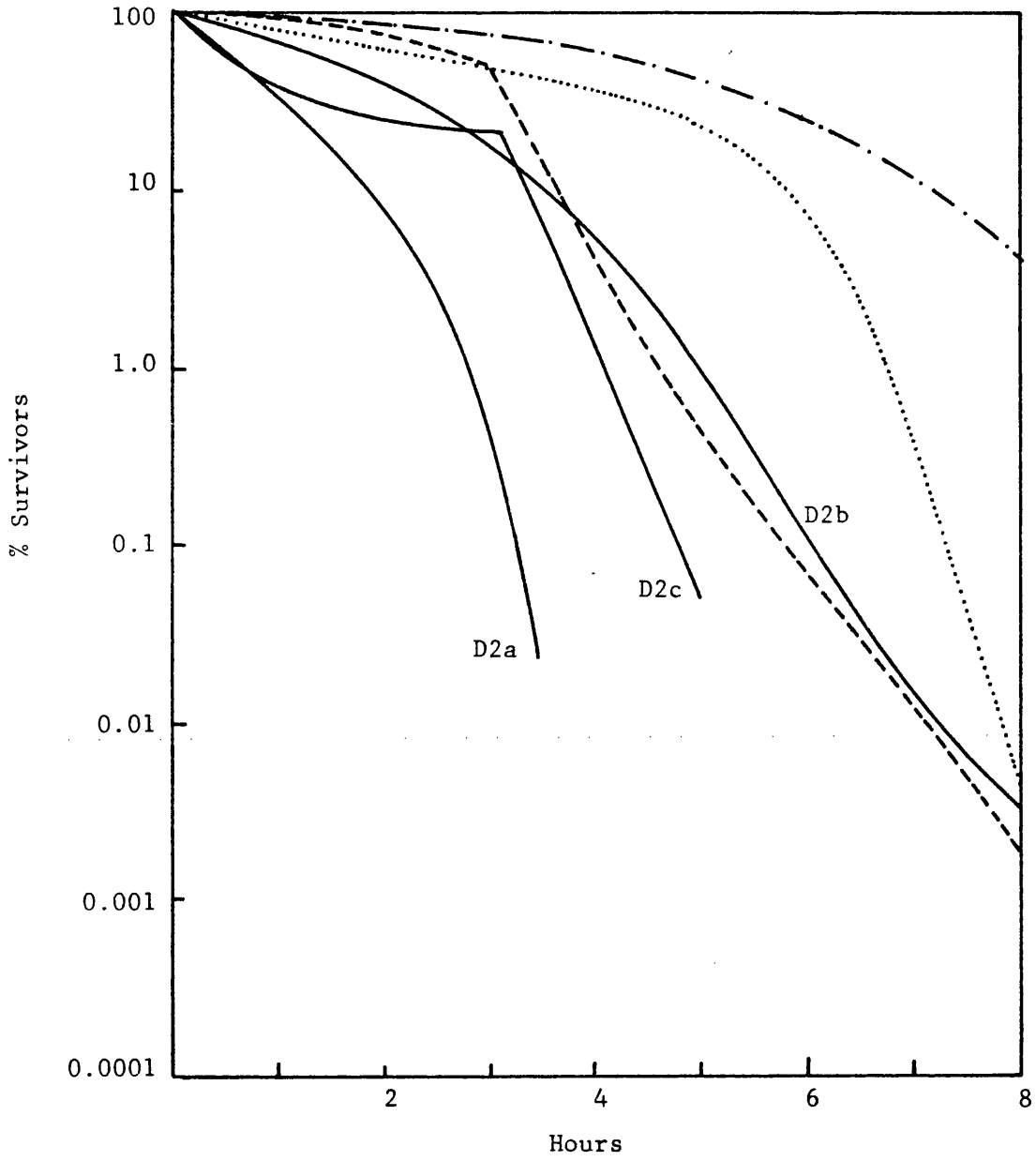


Fig.97 Effect of photodegradation on the antimicrobial activity of thiomersal against E. coli.

(---) Undegraded solution in buffer only

D2a Day-2 degraded solution in buffer only

(·—·) Undegraded solution in buffer with 0.01% w/v EDTA

D2b Day-2 degraded solution in buffer with 0.01% w/v EDTA

(····) Undegraded solution in buffer with 0.1% w/v EDTA

D2c Day-2 degraded solution in buffer with 0.1% w/v EDTA

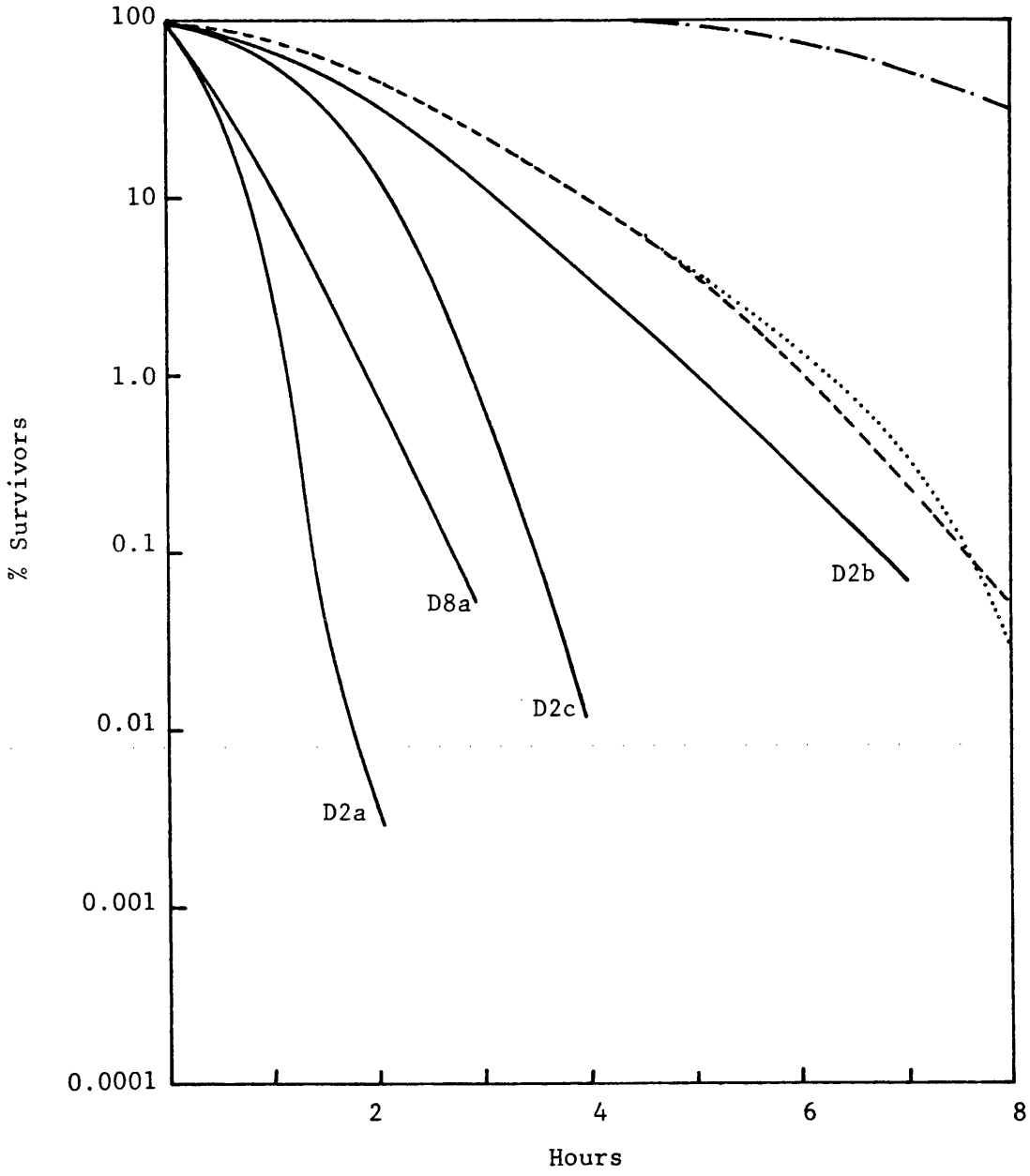


Fig.98 Effect of photodegradation on the antimicrobial activity of thiomersal against C. albicans.

(---) Undegraded solution in buffer only

D2a Day-2 degraded solution in buffer only

D8a Day-8 degraded solution in buffer only

(·—·) Undegraded solution in buffer with 0.01% w/v EDTA

D2b Day-2 degraded solution in buffer with 0.01% w/v EDTA

(····) Undegraded solution in buffer with 0.1% w/v EDTA

D2c Day-2 degraded solution in buffer with 0.1% w/v EDTA

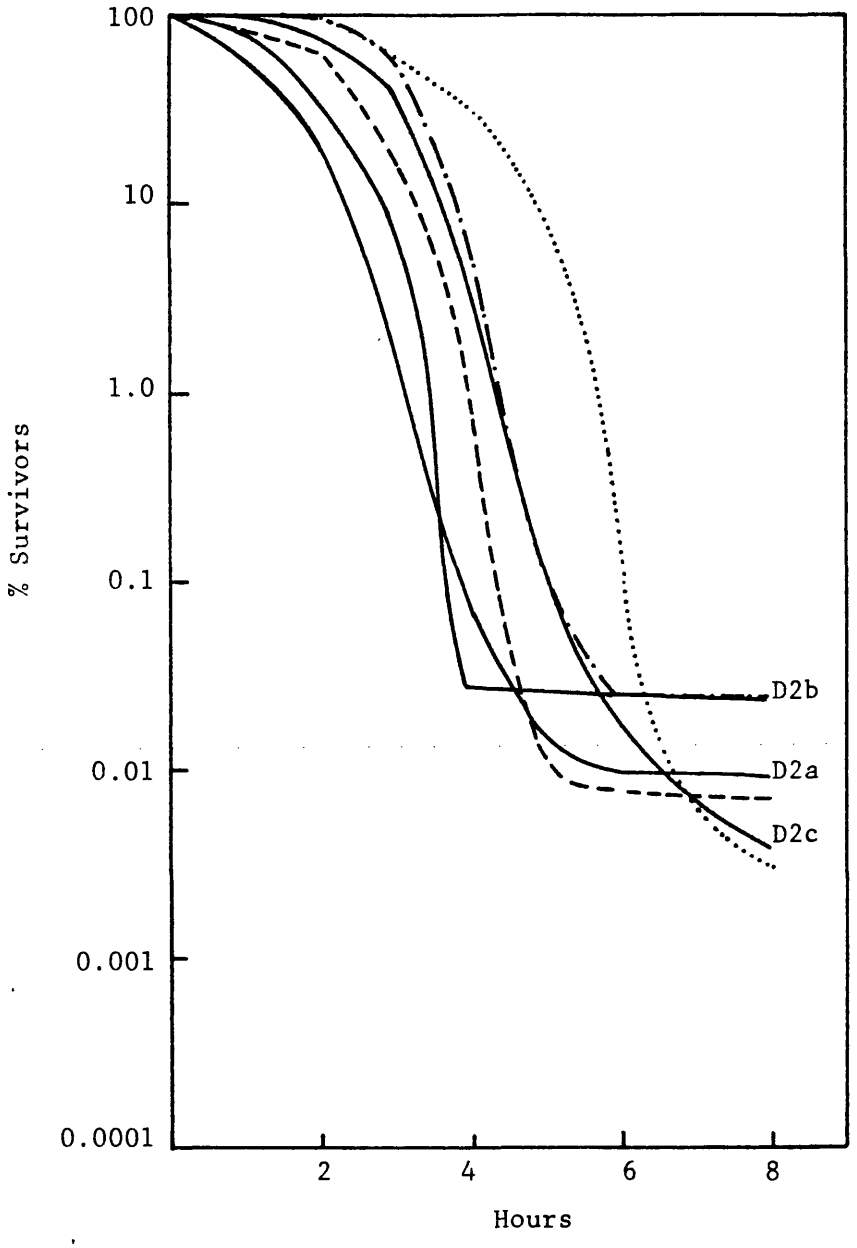


Fig.99 Effect of photodegradation on the antimicrobial activity of thiomersal against B. subtilis.

(---) Undegraded solution in buffer only

D2a Day-2 degraded solution in buffer only

(—·) Undegraded solution in buffer with 0.01% w/v EDTA

D2b Day-2 degraded solution in buffer with 0.01% w/v EDTA

(····) Undegraded solution in buffer with 0.1% w/v EDTA

D2c Day-2 degraded solution in buffer with 0.1% w/v EDTA

thiomersal is present by Day 6 and indeed, very little is available at Day 4, the kill observed must be as a result of an active moiety formed by degradation. The reduced activity seen as compared to degraded solutions without EDTA, does suggest that EDTA is chelating some of the active antimicrobial moiety, less being available for action. Why the Day 10 solution has an increased activity is not clear as the chromatogram for this solution is not very different from that of a Day 8 solution.

When EDTA is included at 0.1% w/v in test solutions that are photochemically degraded, a decrease in activity is seen with all solutions. Figure 95B shows that, although degraded solutions with 0.1% w/v EDTA (represented by lines D2c-D10c), are more active than their undegraded counterpart (represented by a dotted line), an increased holding time in the light box resulted in a decrease in activity, up to and including, Day 8; the Day 10 solution showed an activity similar to that for Days 2 and 4.

If reference is made to the HPLC chromatograms of degraded solutions with 0.1% w/v EDTA (Figures 79c-83, pages 208-212), these show that at this EDTA concentration, the degradation rate of thiomersal is reduced so that some thiomersal is still detectable at Day 10. The numbers and amounts of the degradation products are also reduced and this is reflected in the reduced antimicrobial activity observed. In Figure 95B (page 258), Days 2 and 4 degraded solutions with EDTA at 0.1% w/v only show a slightly enhanced antimicrobial activity in the early stages of the survivor curves, when compared with undegraded solutions without EDTA; Days 6 and 8 degraded solutions with 0.1% w/v EDTA have activities very similar to an undegraded solution with EDTA at 0.01%

w/v (represented by the dash-dot line), and are almost as ineffective as an undegraded solution with EDTA at 0.1% w/v (represented by the dotted line).

The results of the HPLC analyses seem to suggest that, when present at 0.01% w/v, EDTA 'promotes' the degradation of thiomersal. It may do this by preferentially chelating with a degradation product, which consequently disturbs equilibrium between thiomersal and its degraded products; more thiomersal then breaks down to replace the chelated product and the net effect is the acceleration of the degradation process. It may also be possible that at low EDTA concentrations, insufficient EDTA is available to chelate with all of the trace metals present as contaminants in the test solution, that could catalyse the degradation of thiomersal. When present at 0.1% w/v, however, sufficient EDTA would be available to chelate with these so that the net effect is a stabilising of the degradation of thiomersal. This reasoning does seem supported by the very large numbers of degradation product peaks seen in degraded solutions with 0.01% w/v EDTA (Figures 79b-83b, pages 208-212), when compared with those solutions with 0.1% w/v EDTA (Figures 79c-83c, pages 208-212).

Ps. aeruginosa

The data obtained in the photodegradation experiments have been re-plotted diagrammatically in Figures 96A&B, page 259. The figures show that the incorporation of EDTA in solutions prior to photodegradation, resulted in a decrease in the antipseudomonal activity observed in almost all cases, although these solutions were more effective than their undegraded counterparts. An increased holding time in the light box, generally, resulted in a decrease in antipseudomonal activity.

This is clearest with the Day 10 solutions where $t_{1.0}$ values of about 1h, 1h and between 1.5-2.0h were obtained for solutions containing zero, 0.01 and 0.1% w/v EDTA, respectively. Here again, it would appear that EDTA is removing the most active antimicrobial moiety, probably by chelation.

E. coli

The results for this organism have been represented in Figure 97, page 260. Here, Day 2 solutions in buffer, buffer with 0.01% and with 0.1% w/v EDTA, have been selected to represent the net effect of the photodegraded solutions respectively, because of the close proximity of the curves in all cases (Figure 65, page 185; Figure 70, page 192; Figure 75, page 199). The closeness of the curves demonstrated that an increase in photodegradation did not result in a change in antimicrobial activity, at each concentration of EDTA tested. It is interesting that degraded solutions parallel the activity of their undegraded counterparts, for example, when the undegraded solution with 0.01% w/v EDTA (the dash-dot line), was found to be less active than an undegraded solution with 0.1% w/v EDTA (the dotted line), the degraded solution with 0.01% w/v EDTA (line 2b), was also less active than a degraded solution with 0.1% w/v EDTA (line 2c). It may be that the higher EDTA concentration exerts an effect on this organism which contributes to the enhanced kill observed; this, despite the fact that EDTA is probably chelating the active moiety concerned.

C. albicans

A similar effect to that observed with E. coli is seen here (Figure 98, page 261), namely, that degraded solutions with 0.1% w/v EDTA (represented by line 2c), are more effective than degraded solutions with

0.01% w/v EDTA (represented by line 2b). Day 2 solutions have again been selected as representative of the observed antimicrobial effects of degraded solutions containing EDTA on C. albicans, because of the close proximity of these curves (Figure 71, page 193; Figure 76, page 200). Day 2 and Day 8 solutions have been included as examples of the effect of photochemically degraded solutions in buffer only on this organism, as these had different activities (Figure 66, page 186). Quite why solutions with the higher EDTA concentration should have been more active, even when degraded, is not clear. Degraded solutions with EDTA at 0.01% w/v had activities close to those of undegraded solutions without EDTA and with EDTA at 0.1% w/v, so that the effect would seem to be due to the action of EDTA on C. albicans rather than on the solution, supporting the view expressed earlier, that damage to the cell membrane may occur at higher EDTA concentrations.

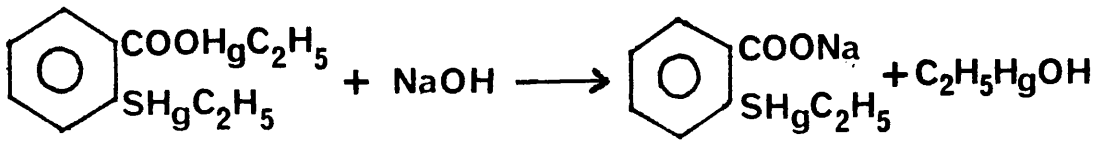
B. subtilis

The data for this organism has been redrawn in Figure 99, page 262, with Day 2 solutions taken as representative of the effects of photo-degradation on all three test formulations. In all cases, degraded solutions were more effective than their undegraded counterparts and here an increase in EDTA concentration generally produced a decrease in antimicrobial activity, which was as anticipated. There is not much difference between the curves obtained though, and when prepared in buffer with 0.1% w/v EDTA, degraded solutions showed a very similar activity to undegraded solutions in buffer only and in buffer with 0.01% w/v EDTA.

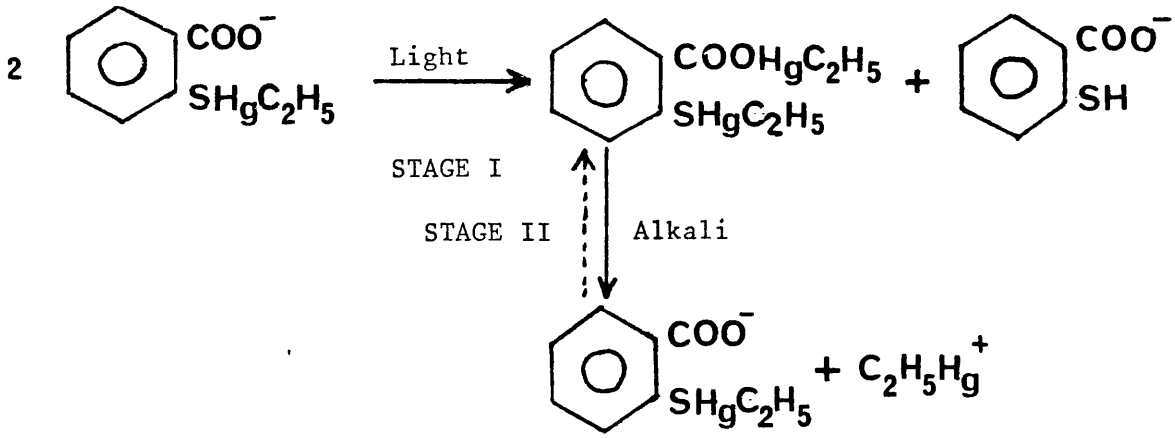
To summarise the results of these antimicrobial tests, it may be said that the inclusion of EDTA in a formulation that undergoes

photochemical degradation generally results in a decrease in the antimicrobial activity of that formulation. This effect was concentration dependant, with the exception of the results obtained for E. coli and C. albicans. Apparently, EDTA is somehow able to remove, from solution, the most active antimicrobial product(s), probably by chelation. A visible phenomenon that occurred with solutions containing EDTA that were photochemically degraded, is the appearance of a grey-black precipitate; the amount of this was observed to increase qualitatively with an increased holding time in the light box and with the EDTA concentration. No such precipitate had been observed in solutions of thiomersal prepared in buffer only. Tanaka amd Mitsuno (97), have isolated a precipitate from degraded solutions of thiomersal, which they identified as being ethylmercuric-2-ethyl-thiomercuric benzoate. Khammas (101) also found that after a 5% aqueous solution of thiomersal had been placed in a light box for 30 days, a black precipitate was obtained; using mass spectrophotometry, he also suggests this was the 'double mercury ester'. It may be that the low concentration of thiomersal used (0.008% w/v) and the short exposure time (10 days), was insufficient for a visible precipitate to be detected, when prepared in buffer only. When EDTA was included in the formulation, it may, by its chelating action, result in a precipitate being formed much earlier.

An interesting outcome of his work is that when the precipitate was dissolved in sodium hydroxide and the hydrolysate chromatographed, a single peak with an identical retention time to that of thiomersal was obtained. A mixture of thiomersal and the hydrolysate also gave a single peak with the retention time of thiomersal. This suggests that the precipitate hydrolyses to give thiomersal as one of the products, according to the following equation:-



The possible effect of EDTA on thiomersal solutions, may be illustrated in the following equation:-



EDTA could stabilise the degradation of thiomersal by removing trace metals that could catalyse the initial photodegradation reaction. Should the reaction at Stage I have occurred, then in the presence of alkali, the Stage II reaction could follow. EDTA could then chelate the $C_2H_5Hg^+$ ion and the net effect would be the generation of more

thiomersal. Thus, a stabilising effect on thiomersal would be seen as indeed was found in the HPLC traces, when EDTA was present at the higher concentration. If, as has been suggested (74), the $C_2H_5Hg^+$ ion is responsible for the observed increase in antimicrobial activity of photochemically degraded solution, then removal of this by chelation could explain the reduction in activity generally observed when EDTA was included in test solutions.

Finally, in an attempt to determine whether the kill observed could be related to a degradation product formed, solutions of thiomersal were assayed by HPLC, both before and after microbial challenge experiments. Table 38 (page 216), contains the results of these analyses, where 0.008% w/v thiomersal had been in contact with Ps. aeruginosa for eight hours. These data show that although a drop in thiomersal peak heights was recorded after the solution had been in contact with the bacteria, this was not statistically significant by the Student 't' test. This was also found to be true for the remaining test organisms (data not shown). No other peak was found in the chromatograms obtained from these solutions, even when the detector sensitivity range was increased.

Photochemically degraded solutions were also analysed by HPLC after microbial challenge experiments, in an attempt to relate the enhanced antimicrobial activity observed in such solutions, with particular peaks or drops in peak heights. The chromatograms generated by these solutions were extremely interesting as they revealed that each degraded solution had itself degraded further, after contact with each test organism. The traces obtained with Day 6 solutions from Staph. aureus and Ps. aeruginosa experiments, are depicted in Figures 85-87,

pages 217-219. Figure 85 illustrates the effect obtained when thiomersal was prepared in buffer without EDTA. Here, no thiomersal is left after 8 hours contact with Ps. aeruginosa, and the degradation product with a retention time at about 3 minutes has increased in height by about two and a half times. After 80 hours contact with Staph. aureus, some thiomersal still remains, but here again, the degradation product at about 3 minutes has increased in height by about three times. This suggests that it has not been entirely formed by the breakdown of thiomersal, as this has occurred to a limited extent only, in the Staph. aureus chromatogram. The heights of some of the other early peaks are much reduced after contact with both bacteria, however, and two peaks occurring after thiomersal are not present in the trace for Staph. aureus.

When EDTA was included in the formulations at 0.01% w/v (Figure 86, page 218), the height of the peak at 3 minutes also increased after contact with both bacteria, though only to a limited extent with Ps. aeruginosa. The numbers and heights of the other peaks were also reduced after contact with both bacteria, especially Staph. aureus, which fits with the increase in peak height of the product with the 3 minute retention time. When EDTA was included in the formulations at 0.1% w/v (Figure 87, page 219), the peak with the retention time at about 3 minutes was again found to increase in height after bacterial contact, this time to a larger extent with Ps. aeruginosa. The thiomersal peak height was reduced in both cases, and the peak with the retention time at about 10 minutes, had markedly dropped in height after contact with both bacteria.

The results indicate that the micro-organisms are themselves degrading the degraded products, or that by attacking a particular product,

they are disrupting any equilibrium between thiomersal and its degradation products, thus causing further degradation to occur. The catabolism of aromatic compounds by micro-organisms is well documented (49, 223-226), and among the bacteria that are active in this respect, are representatives of the families of Coccaceae, Mycobacteriaceae, Pseudomonadaceae, Spirillaceae, Bacteriaceae and Bacillaceae (224). Candida has been reported to grow in media with catechol as sole carbon source (224). In all the above cases of aromatic ring metabolism, molecular oxygen is an obligatory oxidant, and the distinctive biochemical step is ring cleavage. The micro-organisms were being 'starved' in phosphate buffer, and when faced with a potential supply of carbon compounds, had set about degrading these to obtain compounds they could use.

CONCLUSIONS

This research project has demonstrated that experimental factors such as growth medium, composition of test solution, pH, temperature and age of test culture, can markedly affect the antimicrobial activity of undegraded thiomersal. The need for standardisation of such factors must, therefore, be evident, as if the variations obtained here occurred as a result of altering any one of these factors in a single laboratory, it is hardly surprising that inter-laboratory differences arise. A truly meaningful assessment of preservative efficiency can only be made when all possible experimental variables are identified and a standard test established that specifies all such conditions. A panel of test organisms should also be stipulated, supplemented if desired, at the discretion of the individual quality control laboratory.

The variable shape of the survivor curves obtained suggests that the mode of action of thiomersal is rather complex. As any one of a number of sulphydryl group-containing enzymes can be attacked, this is not altogether surprising. At the concentrations at which it is used in contact lens solutions (0.001-0.004% w/v), thiomersal consistently failed to reduce a viable count of 10^6 cfu ml^{-1} of three of the test organisms by three log cycles within six hours at 25°C, thus failing the BP 1980 requirement. The test organisms concerned were Staph. aureus, E. coli and C. albicans. At 20°C, a 0.008% w/v thiomersal solution failed to reduce the viable count of all the test organisms by three log cycles within six hours. The continued use of this organic mercurial in ophthalmic formulations should indeed be questioned.

When photochemically degraded, however, thiomersal solutions were shown to have a greatly improved antimicrobial activity. Although such

solutions still failed to reduce an initial inoculum size of 10^6 cfu ml^{-1} of Staph. aureus by three log cycles within six hours, no survivors were detected at 56 hours, compared with the 0.7% survivors obtained at this time with undegraded solutions. For all the other test organisms, a reduction in viable count of three log cycles was achieved well within six hours. Degraded solutions seemed to be especially effective against Ps. aeruginosa, and here, increased degradation resulted in an increased kill. In view of the serious threat that this organism can pose to the pharmaceutical industry, research to identify the relevant antimicrobial moiety does appear justifiable.

Degraded solutions have been demonstrated to be so much more effective than undegraded solutions, that one must indeed wonder if some of the claims for the efficiency of solutions preserved with thiomersal have been made with such solutions. Photodegradation has been observed to occur extremely quickly, and after about two hours exposure to normal laboratory fluorescent lighting, a degradation peak was detectable by HPLC. As it is unclear, at this moment, what the active moiety is, the implications of this finding may be quite serious. Should the compound concerned be an inorganic mercurial, then it is highly undesirable that such solutions are allowed to come into contact with the eye on a daily basis.

It had been anticipated that the use of differentially-labelled thiomersal would resolve the problem of whether intact thiomersal enters the cell, when it does so, or if it degrades, indicate the active moiety. Unfortunately, the prohibitive cost of even a singly labelled sample made such an investigation impractical. Should suitable finance be available, however, there is no doubt that such studies would be most rewarding.

APPENDIX I

APPENDIX I

Development of a Growth Medium for Staph. aureus NCTC 6571

Introduction

For the reasons outlined on page 64, it had been decided to base the new medium on either M9 or DM. The compositions of these minimal media, together with that of the original growth medium SGM, are given in Table 42.

DM was arbitrarily chosen for the initial experiments, with the omission of sodium citrate as this is a chelating agent, and could also provide a secondary source of carbon. Mah et al (187), had shown that arginine, cysteine and phenylalanine were three amino acids essential for the growth of the strain of Staph. aureus they had investigated. Proline and valine were also found to be necessary when glucose was the carbon source. Eight amino acids that have been suggested as being essential for the growth of Staph. aureus, are cysteine, leucine, valine, proline, glycine, aspartic acid, phenylalanine and arginine. Histidine and methionine were apparently less important and tyrosine and lysine were not required.

Method

It was decided to retain the glucose and vitamins at the same levels as they had been used in SGM and to vary the amino acid composition. Amino acids were therefore added sequentially to the basal medium, in the same concentration as they had been used in SGM, in an attempt to determine which were essential for the growth of this strain of Staph. aureus. The growth flasks were observed daily, and visual readings made, supplemented with optical density readings when appropriate.

Results

The results of these initial growth experiments have been summarised in Table 43.

Discussion

Aspartic acid was found to be critical as once this was included, growth was rapid and luxuriant. Restriction of this amino acid will affect the synthesis of protein directly and indirectly through several other amino acids (lysine, methionine, threonine and isoleucine) and also the synthesis of nucleic acids, mucopeptides and polysaccharides, as shown in the figure below:-

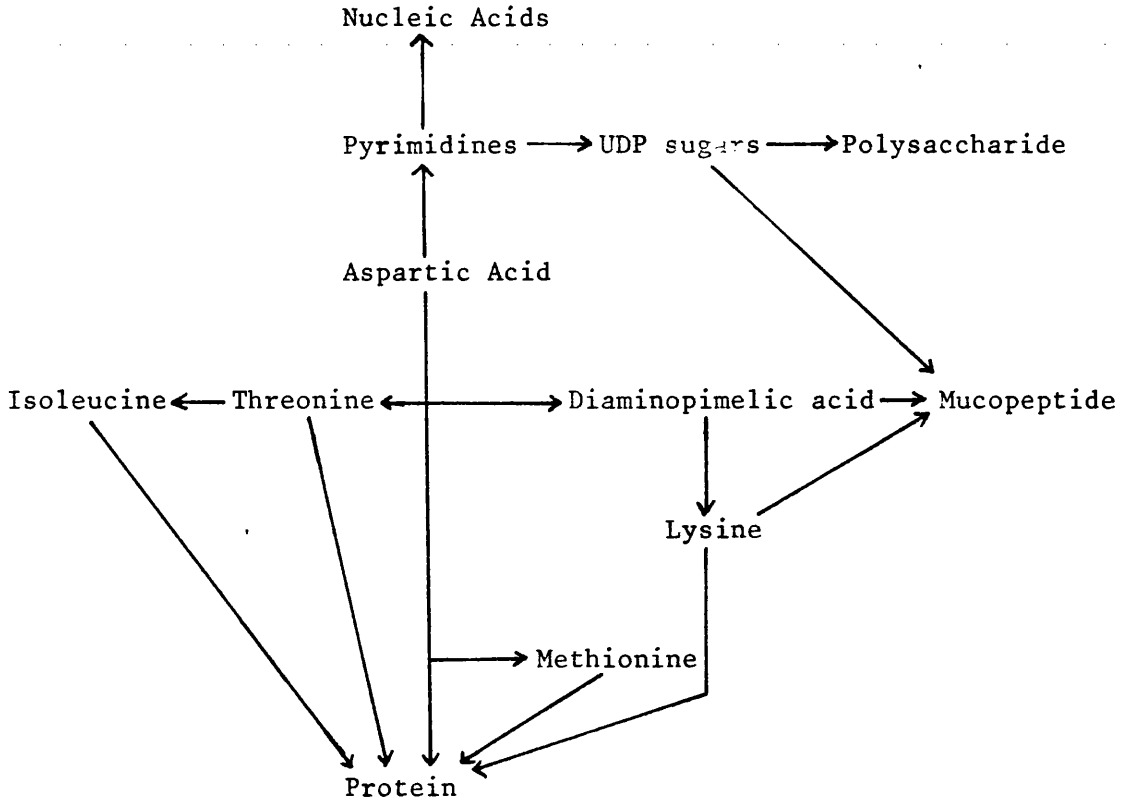


Fig.100 Metabolic interrelationships and control of biosynthesis, (209).

It only remained to decide which minimal salts medium should be used as the new basal medium. Table 42 shows that DM is in fact very

similar to basal SGM, apart from the fact that the ammonium salt used in the former is the sulphate, not the chloride. Moreover, it has been demonstrated that when staphylococci were grown in media containing K_2HPO_4, they showed an increase in viability (227). This is not present in M9. On the basis of these observations, and the fact that DM$^-$ had been proven to be successful, it was selected as the new basal medium.

TABLE 42

Comparison of Inorganic Salts present in SGM, M9 and DM^-

Compound	Concentration in Moles $litre^{-1}$		
	SGM	M9	DM^-
K_2HPO_4	3.21×10^{-2}	-	4.02×10^{-2}
KH_2PO_4	1.76×10^{-2}	2.20×10^{-2}	2.20×10^{-2}
NH_4Cl	9.35×10^{-3}	1.87×10^{-2}	-
$(NH_4)_2SO_4$	-	-	7.57×10^{-3}
$FeSO_4.7H_2O$	3.60×10^{-5}	[a] 1.80×10^{-6}	[a] 1.80×10^{-6}
$MgSO_4.7H_2O$	2.84×10^{-3}	8.11×10^{-4}	4.06×10^{-4}
$Na_2HPO_4.2H_2O$	-	3.37×10^{-2}	-
NaCl	-	8.56×10^{-2}	-

- Not present in formulation

[a] Included as part of possible trace element supplement

TABLE 43

Growth of Staph. aureus NCTC 6571 in a Chemically Defined Medium

Medium Composition	Incubation time at 37°C		
	24h	48h	72h
Basal Medium (BM)	-	-	-
BM + 3AA (Arg, Cys, Phe)	-	-	±
BM + 5AA (Arg, Cys, Phe, Pro, Val)	-	-	±
BM + 7AA (Arg, Cys, Phe, Pro, Val, Leu, Gly)	±	+(0.47)[a]	
BM + 10AA (Arg, Cys, Phe, Pro, Val, Leu, Gly, His, Met, Thr)	±	+(1.14)[a]	
B, + 10AA (Arg, Cys, Phe, Pro, Val, Leu, Gly, His, Met, Asp)	+(1.22)[a]		

Basal Medium: DM^- + Glucose (1%) + vitamin supplement (page 64)

AA : Amino Acid

\- : No visible growth

± : Medium faintly cloudy with small aggregates

\+ : Medium turbid (O.D. readings made at 470nm with BM as blank)

a : Experiment stopped

APPENDIX II

APPENDIX II

Development of Modified Recovery Medium

Introduction

As has been reviewed in the introduction to this thesis, (page 43), sodium thioglycollate is required as an inactivator for thiomersal. The recovery medium used by Norton *et al* (53), included this compound at a concentration of 0.1% w/v. Preliminary plating experiments using this level of thioglycollate and a relatively high concentration of thiomersal (0.01% w/v), revealed a 'zoning' phenomenon when *Staph. aureus* was the test organism (See Results-part A, below).

Elkhouly and Yousef had shown in 1974 that the percentage of survivors they obtained was influenced by the thioglycollate concentration, which in turn varied with the test organism (179). For *Ps. aeruginosa*, the optimum concentration was shown to be 0.2%, whereas for *Staph. aureus*, it was 2-3%. It was decided therefore to investigate the effect of varying the thioglycollate concentration on the recovery of exposed organisms.

Methods

Recovery media were prepared which contained sodium thioglycollate at 1%, 2% and 3% w/v. *Staph. aureus* was the challenge organism used. The concentration of thiomersal used was 0.01% w/v, prepared in DM^{-}. All dilutions were carried out in this diluent too. A challenge experiment was set up using the technique described on page 79. A sample was withdrawn after the bacteria had been in contact with the thiomersal for one hour, directly into the appropriate recovery media and left for 10 minutes. All dilutions were plated to see which gave the best recovery. The plates were incubated at $37^{o}C$ and examined daily.

Results-A

These results are of the preliminary experiments and describe the 'zoning' phenomenon obtained.

TABLE 44

Recovery medium with 0.1% thioglycollate

Dilution plated	Viable count after 24h at 37°C			
-1 (RM only)	0	0	0	0
-2 (RM + tenfold dilution)	Uc‡‡	Uc	Uc	Uc
-3 (RM + two tenfold dilutions)	52	51	54	47
-3 (Control, three tenfold dilutions)	50	57	51	54

Uc‡‡ Uncountable plate

Observations at 24 hours. The -3 dilution plates showed typical staphylococcal colonies, distributed uniformly over the agar surface. The -2 dilution plates showed a larger number of colonies, which, as anticipated, were too numerous to count. These colonies were chiefly distributed around the circumference and towards the centre of the plate; the central region of the plates had no colonies, however. The -1 dilution plates which should have had confluent growth, had no colonies at all.

Observations at 48 hours. The -3 dilution plates were unchanged apart from an increase in the size of the colonies.

With the -2 dilution plates, growth had now occurred over the entire plate.

With the -1 dilution plates, a few colonies had appeared around the circumference of the plates.

Observations at 72 and 96 hours. These remained unchanged from those observed at 48 hours.

Results-B

TABLE 45

Recovery Medium with 1% thioglycollate

Dilution plated	Viable count after 24h at 37°C			
-1	Uc	Uc	Uc	Uc
-2	Uc	Uc	Uc	Uc
-3	70	74	77	69

TABLE 46

Recovery Medium with 2% thioglycollate

Dilution plated	Viable count after 24h at 37°C			
-1	Uc	Uc	Uc	Uc
-2	Uc	Uc	Uc	Uc
-3	67	76	61	73

TABLE 47

Recovery Medium with 3% thioglycollate

Dilution plated	Viable count after 24h at 37°C			
-1	Uc	Uc	Uc	Uc
-2	Uc	Uc	Uc	Uc
-3	76	69	71	63
-3(Control, no RM)	70	70	73	79

Discussion

The results in part A suggest that the RM was not allowing full recovery of the cells. Growth at the -3 dilution occurred at 24 hours, presumably because the thiomersal had been so diluted out that it was no longer inhibitory. The possibility that the 10 minute incubation time in the RM was insuffient was ruled out when an incubation time of 30 minutes was tried with no effect.

The results in part B show that raising the thioglycollate concentration to 1% was sufficient for the inactivation of 0.01% thiomersal with Staph. aureus. This requirement for 1% w/v thioglycollate to inactivate 0.01% thiomersal was later confirmed by HPLC analysis. The peak for thiomersal disappeared after momentary contact with 1% thioglycollate (see page 221).

For the sake of standardisation, all recovery media were now prepared with 1% w/v sodium thioglycollate, regardless of the test organism. Tests for the efficacy of this recovery medium are provided in the Materials and Methods (pages 71-74).

APPENDIX III

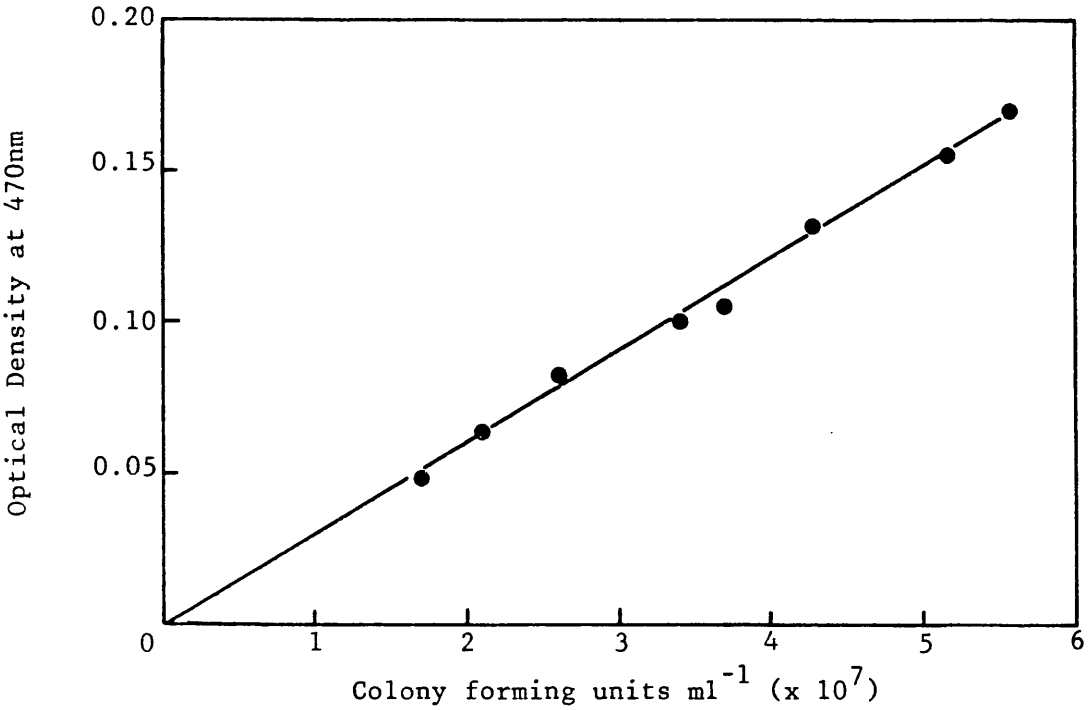


Fig. 1 Relationship between optical density and viable count for Staph. aureus NCTC 6571, grown for 22h at 37°C in a chemically-defined medium.

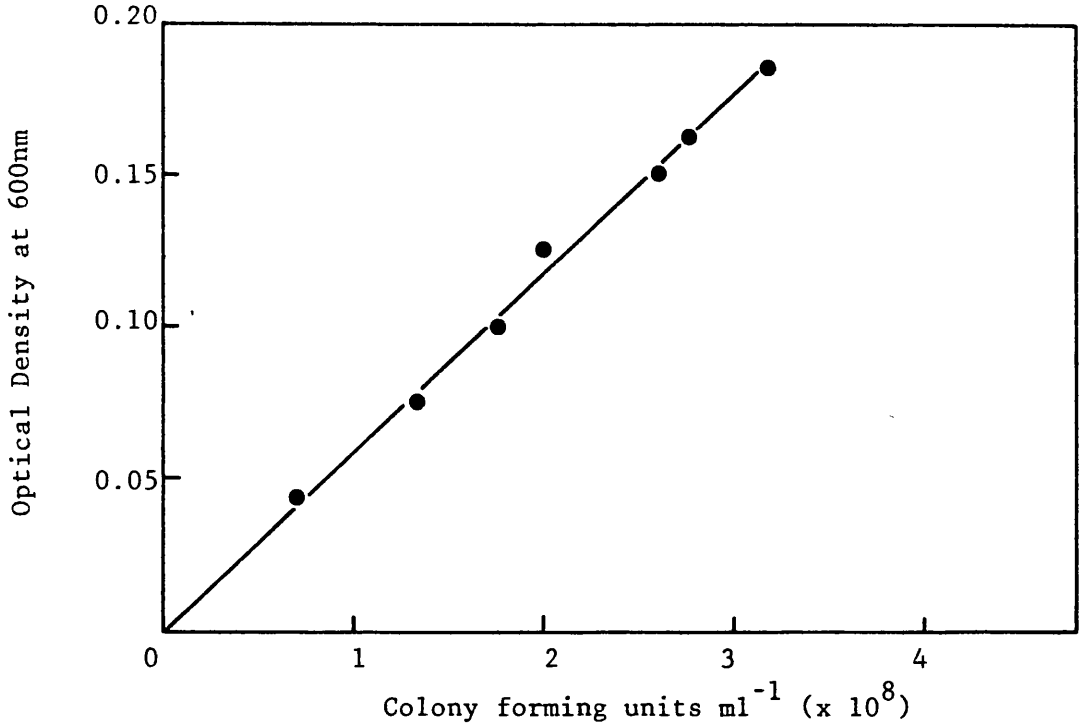


Fig.2 Relationship between optical density and viable count for Ps. aeruginosa NCTC 6750, grown for 22h at 37°C in a chemically-defined medium.

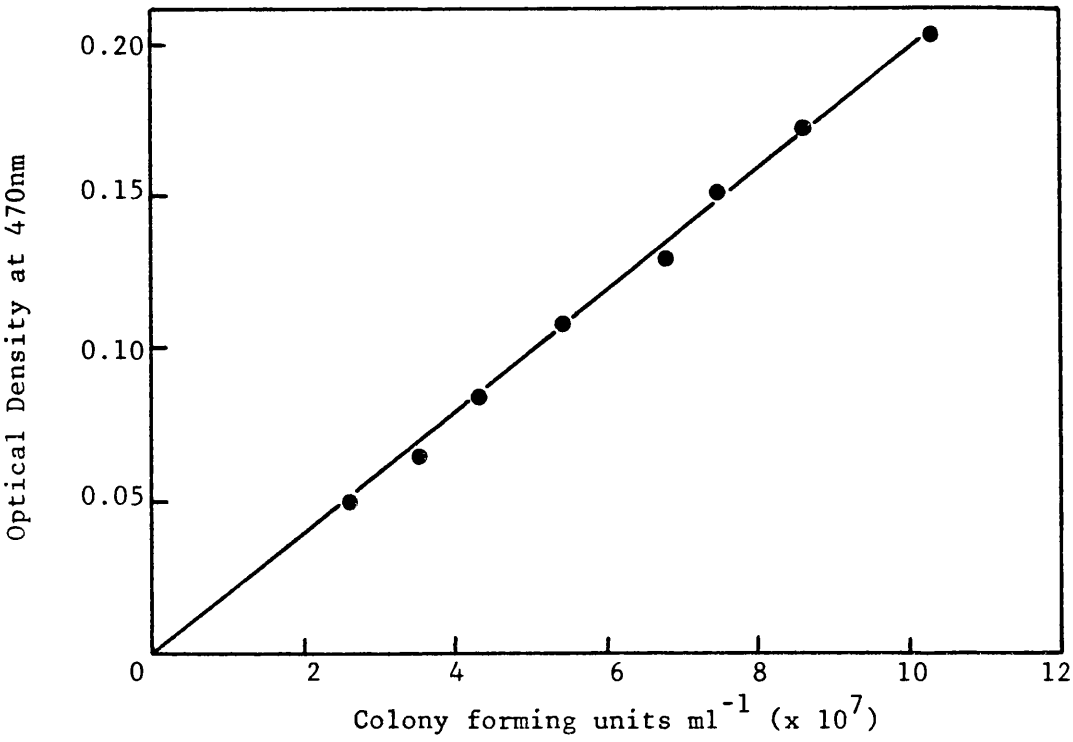


Fig.3 Relationship between optical density and viable count for E. coli NCTC 86, grown for 22h at 37°C in a chemically-defined medium.

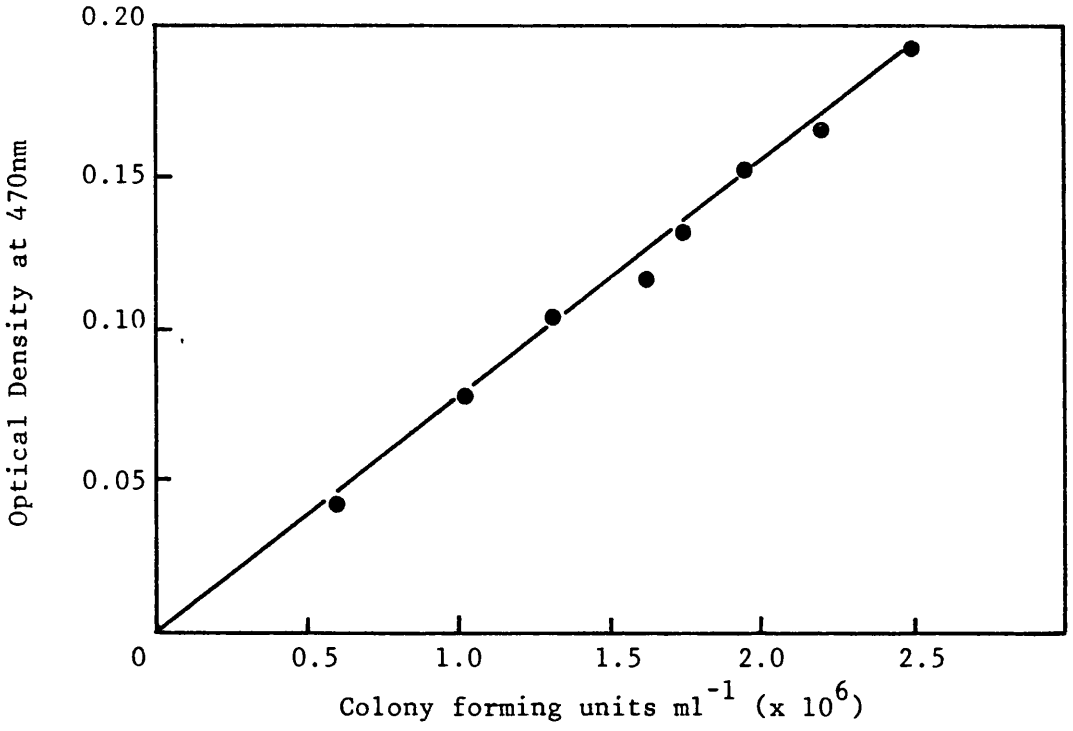


Fig.4 Relationship between optical density and viable count for C. albicans LSHTM 3153, grown for 22h at 37°C in a chemically-defined medium.

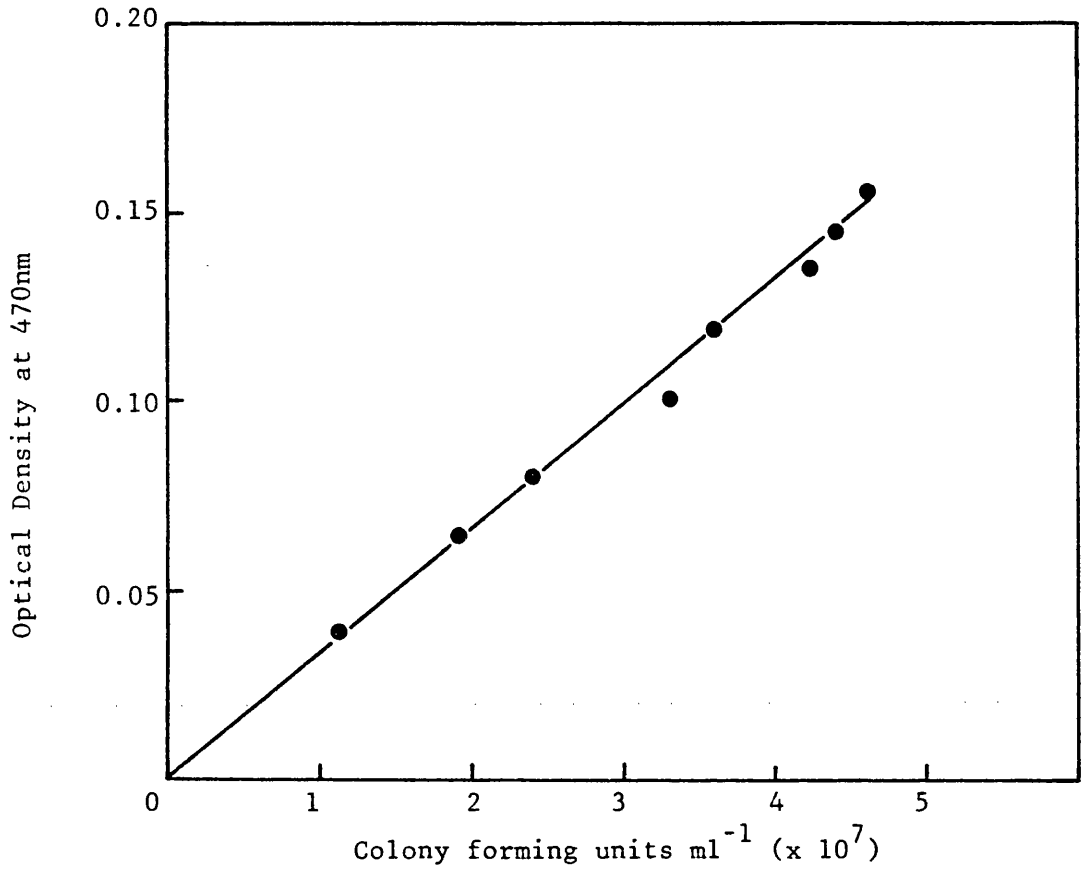


Fig.5 Relationship between optical density and viable count for B. subtilis FD TEMP, grown for 22h at 37°C in a chemically-defined medium.

APPENDIX IV

APPENDIX IV

Development of an HPLC Assay for Thiomersal in the Presence of its Photodegradation Products

Introduction

Methods are available in the literature for the HPLC analysis of undegraded solutions of thiomersal (228). It was decided to develop a system for the detection of thiomersal in the presence of its photodegradation products, using reversed-phase, ion-pair chromatography.

In ion-pair chromatography, the pH of the mobile phase is adjusted so that ionisation is effected. A strong electrolyte, such as the tetra alkyl ammonium compounds, is then added. The ionised analyte and the added counterion form a reversible complex ($RCOO^{-}...R_4N^{+}$), which has great lipophilicity and may thus partition into the hydrophobic stationary phase. Retention may be influenced by any of the following factors (229):-

1) The Counterion

Type. The better the ability to pair, the longer the retention.

Size. An increase in the size of the counterion will increase retention.

Concentration. An increase in concentration will increase retention, up to a limit.

2) pH

This effect is dependant upon the nature of the solute. For greatest retention, maximum ionisation is desirable; consideration must be given, however, to the stability of the column packing, and optimum performance and lifetime is achieved when the pH is in the range of 2-7.4 pH units.

3) Organic Modifier

Type. Retention decreases with an increase in lipophilic nature.

Concentration. Retention decreases with an increase in concentration.

4) Temperature

Retention decreases with an increase in temperature.

5) Stationary Phase

Retention increases with an increase in lipophilicity or a higher degree of coverage.

Method

Using Acetonitrile as the organic modifier, CTAB as the counterion, double-strength Sörensen's phosphate buffer and the column and packing materials described (page 90), the effects of modifications of the acetonitrile and the CTAB concentration, pH, temperature and wavelength were determined on the separation of thiomersal and its photodegraded products. A stock solution of thiomersal at 0.04% w/v in isotonic Sörensen's phosphate Buffer (pH 7.0) was photochemically degraded for 13 days and used in these experiments after an initial 1:10 dilution had been carried out.

Results

These have been summarised as shown below.

1) Increasing CTAB Concentration

Mobile Phase

Acetonitrile	43%
2/15M Sörensen's phosphate buffer, pH 5.8	10%
1×10^{-2}M CTAB	15%
Water	to 100%

Experimental Conditions

Flow rate : 2ml min^{-1}

Detector : uv at 230nm

Temperature: $30^{o}C$

Effect on Chromatogram

Increasing the CTAB concentration resulted in poor separation of the peaks.

2) Reducing CTAB Concentration

Mobile Phase

CH_3CN	40%
2/15M Sörensen's phosphate buffer, pH 5.8	10%
1×10^{-2}M CTAB	7%
0.5ml n/10 NaOH	

Experimental Conditions

Flow rate : 2ml min^{-1}

Detector : uv at 230nm

Temperature: 30°C

Effect on Chromatogram

Reducing the CTAB concentration resulted in a reduction in the retention times of all peaks, and poor separation was obtained.

3) Reducing CTAB and Acetonitrile Concentration

Mobile Phase

Acetonitrile	37%
2/15M Sörensen's phosphate buffer, pH 5.8	10%
1×10^{-2}M CTAB	7%
0.5ml N/10 NaOH	

Experimental Conditions

Flow rate : 2ml min^{-1}

Detector : uv at 230nm

Temperature: 30°C

Effect on Chromatogram

Reducing both counterion and organic modifier concentration resulted in a reduction in the retention times for the initial peaks up to and

including thiomersal; good resolution was obtained with these peaks, but poor separation was obtained with peaks occurring after the thiomersal peak.

4) Increasing Temperature to 40°C and keeping the CTAB Concentration High

Mobile Phase

Acetonitrile	43%
2/15M Sörensen's phosphate buffer, pH 5.8	10%
1×10^{-2}M CTAB	15%

Experimental Conditions

Flow rate : 2.2ml min^{-1}

Detector : uv at 230nm

Temperature: 40°C

Effect on Chromatogram

Increasing the temperature decreased the retention time markedly and poor separation of the peaks after thiomersal was obtained.

5) Changing Wavelength to 240nm and 250nm

Mobile Phase

Acetonitrile	43%
2/15M Sörensen's phosphate buffer, pH 5.8	10%
1×10^{-2}M CTAB	12%
Water	to 100%

Experimental Conditions

Flow rate : 2ml min^{-1}

Detector : uv at 240nm and 250nm

Temperature: 30°C

Effect on Chromatogram

Increasing the wavelength resulted in a decrease in the peak heights obtained for thiomersal and all its degradation products.

6) Increasing pH of Mobile Phase to 7.0

Mobile Phase

Acetonitrile	40%
2/15M Sörensen's phosphate buffer, pH 5.8	10%
1×10^{-2}M CTAB	12%
0.5ml N/10 NaOH (to raise the pH to 7.0)	
Water	to 100%

Experimental Conditions

Flow rate : 2ml min^{-1}

Detector : uv at 230nm

Temperature: 30°C

Effect on Chromatogram

Good separation of the initial degradation products was obtained, but poor separation of thiomersal from its adjacent degradation products was found.

Finally, the best separation was found when the following mobile phase and conditions were used:-

Mobile Phase

Acetonitrile	38%
1×10^{-2}M CTAB	12%
2/15M Sörensen's phosphate buffer, pH 5.8	10%
Water	to 100%

Experimental Conditions

Flow rate : 2.2ml min^{-1}

Detector : uv at 235nm

Temperature: 30°C

Discussion

When the above mobile phase and conditions were used, adequate separation of thiomersal from its degradation products was obtained. This system was developed for thiomersal, photochemically degraded in isotonic Sörensen's phosphate buffer (pH 7.0); if another diluent is used, further modifications will be necessary.

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